

FORM PTO-1390  
(REV. 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

6550-000038/CPB

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)

09/937243

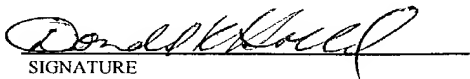
INTERNATIONAL APPLICATION NO. PCT/US00/0608	INTERNATIONAL FILING DATE 16 March 20	PRIORITY DATE CLAIMED 23 March 1999
TITLE OF INVENTION Synthesis of 1,2,3,4-Tetrahydroxybenzenes and 1,2,3-Trihydroxybenzenes Using Myo-Inositol-1 Phosphate Synthase and Myo-Inositol 2-Dehydrogenase		
APPLICANT(S) FOR DO/EO/US John W. Frost, Chad Hansen		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ has been communicated by the International Bureau.
  - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

## Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:  
Express Mailing (37 CFR 1.10(c)) No. EL869991894US  
check in the amount of \$528.00

U.S. APPLICATION NO. <b>09/937243</b> INTERNATIONAL APPLICATION NO.		ATTORNEY'S DOCKET NUMBER	
21. <input checked="" type="checkbox"/> The following fees are submitted: <b>BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b>  International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b>  International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... <b>\$100.00</b>  <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		<b>CALCULATIONS PTO USE ONLY</b>	
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$ 100.00 \$ 130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	57 - 20 =	37	x \$18.00
Independent claims	5 - 3 =	2	x \$80.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$270.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>		\$1056.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.		\$ 528.00	
<b>SUBTOTAL =</b>		\$	
Processing fee of <b>\$130.00</b> for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		\$	
<b>TOTAL NATIONAL FEE =</b>		\$ 528.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property +		\$	
<b>TOTAL FEES ENCLOSED =</b>		\$ 528.00	
		Amount to be refunded:	\$
		charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>528.00</u> to cover the above fees is enclosed.  b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>08-0750</u> . A duplicate copy of this sheet is enclosed.  d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card</b> <b>information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038.			
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.</b>			
SEND ALL CORRESPONDENCE TO  <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">           Harness Dickey &amp; Pierce PLC            7700 Bonhomme Ave., STe. 400            St. Louis, MO 63105         </div> <div style="width: 45%; text-align: right;">             SIGNATURE             Donald R. Holland            NAME   <u>35,197</u>            REGISTRATION NUMBER         </div> </div>			

09/937243

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Express Mail No.: EL869992894US

Date of Deposit: September 21, 2001

Application No.: unknown

Filing Date: March 16, 2000

Applicant: FROST

Group Art Unit: unknown

Examiner: unknown

Title: **SYNEHESIS OF 1,2,3,4-TETRA-HYDROXYBENZENES AND  
1,2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1,  
PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DEHYDROG-  
GENASE**

Attorney Docket: 6550-000038/CPB

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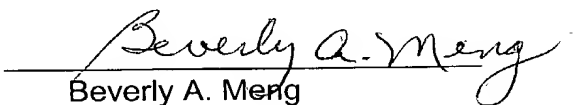
Box PCT  
Hon. Commissioner of Patents and Trademarks  
Washington, D.C. 20231

**EXPRESS MAIL TRANSMITTAL**

The following papers are being deposited with the United States Postal Service Express Mail Post Office To Addressee and addressed for receipt by the United States Patent and Trademark Office:

1. Transmittal letter to the U.S. Designated/Elected Office
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HARNES, DICKEY & PIERCE, P.L.C.  
7700 Bonhomme, Ste. 400  
St. Louis, Missouri 63105  
(314) 726-7500

  
Beverly A. Meng

09/937243

PATENT

Attorney Docket No. 6550-000038

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Frost	:	
	:	
Serial No: Unknown	:	Art Unit:
	:	
National Stage Filed: September 21, 2001	:	Examiner:
	:	
For: SYNEHESIS OF 1,2,3,4-TETRA-	:	
HYDROXYBENZENES AND	:	
1,2,3-TRIHYDROXYBENZENES	:	
USING MYO-INOSITOL-1	:	
PHOSPHATE SYNTHASE AND	:	
MYO-INOSITOL 2-DEHYDROG-	:	
GENASE	:	

Assistant Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

This paper is submitted as a preliminary amendment. Please cancel claims 1-57 without prejudice or disclaimer and enter new claims 58-103 in accordance with the following rewritten claims in clean form prior to examination on the merits.

AMENDED CLAIMS REWRITTEN IN CLEAN FORM:

58. (New) A method for the production of 1,2,3,4-tetrahydroxybenzene, comprising:

a) incubating, in the presence of a carbon source, a first microbe comprising a recombinant DNA encoding *myo*-inositol-1-phosphate synthase and a second microbe which expresses inositol dehydrogenase activity to produce *myo*-2-inosose; and

b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

59. (New) The method of claim 58 wherein the first microbe comprises *INO1*.

60. (New) The method of claim 59 wherein the *INO1* gene comprises an *Saccharomyces cerevisiae INO1*.

61. (New) The method of claim 60 wherein *INO1* comprises pAD1.88A.

62. (New) The method of claim 58 wherein the first microbe is an *Escherichia coli*.

63. (New) The method of claim 62 wherein the *Escherichia coli* is JWF1/pAD1.88A.

64. (New) The method of claim 58 wherein the second microbe is *Gluconobacter oxydans*.

65. (New) The method of claim 64 wherein the *Gluconobacter oxydans* is ATCC 621.

66. (New) The method of claim 58 wherein the second microbe comprises a recombinant DNA encoding inositol dehydrogenase.

67. (New) The method of claim 58 wherein the DNA encoding inositol dehydrogenase comprises a *Bacillus subtilis iolG* gene.

68. (New) The method of claim 58 wherein the carbon source comprises glucose.

69. (New) A method for the production of 1,2,3-trihydroxybenzene, comprising producing 1,2,3,4-tetrahydroxybenzene in accordance with claim 1 and reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

70. (New) A method for the production of 1,2,3,4-tetrahydroxybenzene, comprising:  
a) incubating, in the presence of a carbon source, a microbe comprising a first recombinant DNA encoding *myo*-inositol-1-phosphate synthase and a second recombinant DNA encoding inositol dehydrogenase, to produce *myo*-2-inosose; and  
b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

71. (New) The method of claim 70 wherein the recombinant DNA encoding *myo*-inositol-1-phosphate synthase comprises *INO1*.

72. (New) The method of claim 71 wherein *INO1* comprises a *Saccharomyces cerevisiae* *INO1*.

73. (New) The method of claim 70 wherein the DNA encoding inositol dehydrogenase comprises *iolG*.

74. (New) The method of claim 70 wherein the DNA encoding inositol dehydrogenase comprises a *Bacillus subtilis* *iolG*.

75. (New) The method of claim 70 wherein the first recombinant DNA encoding *myo*-inositol-1-phosphate synthase and the second recombinant DNA encoding inositol dehydrogenase comprise pAD2.88A.

76. (New) The method of claim 70 wherein the microbe is an *Escherichia coli*.

77. (New) The method of claim 70 wherein the carbon source comprises glucose.

78. (New) A method for the production of 1,2,3-trihydroxybenzene, comprising producing 1,2,3,4-tetrahydroxybenzene in accordance with claim 70 and reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

79. (New) A microbe comprising a recombinant DNA encoding *myo*-inositol-1-phosphate synthase.

80. (New) The microbe of claim 79 wherein the recombinant DNA encoding *myo*-inositol-1-phosphate synthase comprises *INO1*.

81. (New) The microbe of claim 80 wherein *INO1* comprises a *Saccharomyces cerevisiae* *INO1*.

82. (New) The microbe of claim 81 wherein *INO1* comprises pAD1.88A.

83. (New) The microbe of claim 79 which is an *Escherichia coli*.

84. (New) The microbe of claim 83 wherein the *Escherichia coli* is JWF1/pAD1.88A.

85. (New) The microbe of claim 79 further comprising a recombinant DNA encoding inositol dehydrogenase.

86. (New) The microbe of claim 85 wherein the recombinant DNA encoding inositol dehydrogenase comprises a *Bacillus subtilis* *iolG* gene.

87. (New) A fermentation composition comprising a first microbe which comprises a recombinant DNA encoding *myo*-inositol-1-phosphate synthase and a second microbe which expresses inositol dehydrogenase.

88. (New) The fermentation composition of claim 87 wherein the first microbe comprises an *INO1* gene.

89. (New) The fermentation composition of claim 88 wherein the *INO1* gene comprises a *Saccharomyces cerevisiae INO1* gene.

90. (New) The fermentation composition of claim 89 wherein the *INO1* gene comprises pAD1.88A.

91. (New) The fermentation composition of claim 87 wherein the first microbe is an *Escherichia coli*.

92. (New) The fermentation composition of claim 91 wherein the *Escherichia coli* is JWF1/pAD1.88A.

93. (New) The fermentation composition of claim 87 wherein the second microbe is *Gluconobacter oxydans*.

94. (New) The fermentation composition of claim 93 wherein the *Gluconobacter oxydans* is ATCC 621.

95. (New) The fermentation composition of claim 87 wherein the second microbe comprises a recombinant DNA encoding inositol dehydrogenase.

96. (New) The fermentation composition of claim 95 wherein the DNA encoding inositol dehydrogenase comprises a *Bacillus subtilis iolG* gene.

97. (New) The fermentation composition of claim 87 further comprising glucose.



98. (New) A fermentation composition comprising a microbe which comprises a first recombinant DNA encoding *myo*-inositol-1-phosphate synthase and a second recombinant DNA encoding inositol dehydrogenase.

99. (New) The fermentation composition of claim 98 wherein the recombinant DNA encoding *myo*-inositol-1-phosphate synthase comprises an *INO1* gene.

100. (New) The fermentation composition of claim 99 wherein the *INO1* gene comprises a *Saccharomyces cerevisiae* *INO1* gene.

101. (New) The fermentation composition of claim 100 wherein the *INO1* gene comprises pAD1.88A.

102. (New) The fermentation composition of claim 98 wherein the microbe is an *Escherichia coli*.

103. (New) The fermentation composition of claim 98 wherein the DNA encoding inositol dehydrogenase comprises a *Bacillus subtilis* *iolG* gene.

104. (New) The fermentation composition of claim 98 further comprising glucose.

### **Remarks**

This paper cancels claims 1-57 and adds new claims 58-103 so that claims 58-103 remain pending in the case. No new matter is believed to be added in the amendments to the claims. Support for the new claims can be found in the specification as follows:

Claim 58 finds support in originally filed claim 1. Reference to "incubating, in the presence of a carbon source, a first microbe" finds support at least on page 7, line 25 through page 8, line 2. Reference to a microbe comprising recombinant DNA encoding

*myo*-inositol-1-phosphate synthase can be found at least in claim 2 as originally filed.

Reference to a microbe which expresses inositol dehydrogenase activity to produce *myo*-2-inosose can be found at least in claim 7 as originally filed and on page 5 lines 3-15.

Claims 59 and 60 find support at least in claims 3 and 4 as originally filed.

Claim 61 finds support at least in claim 5 as originally filed.

Claims 62 and 63 are supported at least by claim 6 as originally filed, on page 2, lines 20-22 and on page 6, lines 19-24.

Claims 64 and 65 are supported at least by claims 8 and 9 as originally filed.

Claims 66 and 67 are supported at least by claims 10 and 11 as originally filed and on page 5, lines 4-10.

Claim 68 is supported at least by claim 12 as originally filed.

Claim 69 is supported at least by claim 13 as originally filed and on page 3, lines 27-32.

Claim 70 is supported at least by claims 20, 21 and 30 as originally filed, on page 7, line 25 through page 8, line 2 and on page 22, lines 5-17.

Claim 71 and 72 are supported at least by claims 21 and 22 as originally filed.

Claims 73 and 74 are supported at least by claims 25 and 26 as originally filed.

Claim 75 is supported at least by claims 24 and 28 as originally filed and on page 6, lines 17-18.

Claim 76 is supported at least on page 2, lines 20-22 and on page 6, lines 19-24.

Claim 77 is supported at least on page 4, lines 14-17 and lines 25-28.

Claim 78 is supported at least on page 3, lines 30-33.

Claim 79 is supported at least on page 4, lines 29-34.

Claims 80 and 81 are supported at least on page 4, lines 31-35.

Claim 82 is supported at least on page 4, lines 34-35.

Claim 83 and 84 are supported on page 5, lines 16-17, page 6, lines 19-24 and page 9, lines 13-17 and lines 24-26.

Claims 85 and 86 are supported at least on page 5, lines 3-10.

In claim 87, reference to a fermentation composition can be found at least on page 11, lines 25-33. Reference to a microbe comprising a recombinant DNA encoding *myo*-inositol-1-phosphate synthase can be found at least on page 4, lines 29-34. Reference to

a microbe which expresses inositol dehydrogenase can be found at least on page 5, lines 9-12.

Claims 88 and 89 are supported at least on page 4, lines 31-35.

Claim 90 is supported on page 11, line 25-page 12, line 10.

Claims 91 and 92 are supported on page 5, lines 16-17, page 6, lines 19-24 and page 9, lines 13-17 and lines 24-26.

Claims 93 and 94 are supported at least on page 11, lines 25-33, and page 14, line 33-page 15, line 4.

Claims 95 and 96 are supported at least on page 5, lines 3-10.

Claim 97 is supported at least on page 4, lines 14-17 and lines 25-28.

In new Claim 98, reference to a fermentation composition can be found at least on page 11, lines 25-33; reference to recombinant DNA encoding *myo*-inositol-1-phosphate synthase can be found at least on page 4, lines 31-34; page 5, lines 16-20; page 9, lines 17-19; and page 12, line 19-page 13, line 6. Reference to recombinant DNA encoding inositol dehydrogenase can be found at least on page 5, lines 4-10. Reference to a microbe comprising a first recombinant DNA encoding *myo*-inositol-1-phosphate synthase and a second recombinant DNA encoding inositol dehydrogenase can be found at least on page 17, lines 17-20 and lines 26-30, and page 22, lines 6-17.

Claim 98 is supported at least on page 6, lines 14-29 and on page 22, lines 5-18.

Claims 99 and 100 are supported at least on page 4, lines 31-35.

Claim 101 is supported on page 11, line 25-page 12, line 10.

Claim 102 are supported on page 4, lines 29-30; page 5, lines 16-17, page 6, lines 19-24 and page 9, lines 13-17 and lines 24-26.

Claim 103 is supported at least on page 5, lines 3-10.

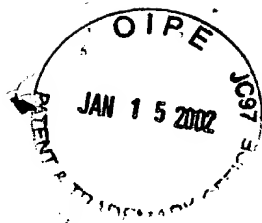
Claim 104 is supported at least on page 4, lines 14-17 and lines 25-28 and on page 11, lines 28-31.

It is requested that the amendments above be entered and that the claims be examined on the merits. Should any questions arise, the Patent and Trademark Office is requested to contact the undersigned attorney.

Respectfully submitted,



Donald R. Holland, Reg. No. 35,197  
HARNESSE, DICKEY & PIERCE, P.L.C.  
7700 Bonhomme  
Suite 400  
St. Louis, Missouri 63105  
(314) 726-7519



JCO3 Rec'd 15 JAN 2002

PCT\$ #

PATENT

Attorney Docket No. 6550-000038/CPB

09/937243

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Frost ✓

Serial No: Unknown

National Stage Filed: September 21, 2001

From: PCT/US00/06808

Filed: March 16, 2000

For: **SYNTHESIS OF 1,2,3,4-TETRA-HYDROXYBENZENES AND 1,2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1 PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DEHYDRO-GENASE**

Art Unit:

Examiner:

Box Non-Fee Amendment  
Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT B

This paper is submitted as a Preliminary Amendment B. Please enter amended claim 69 and new claims 105-116 in accordance with the following rewritten claims in clean form prior to examination on the merits. Applicant includes herewith an Attachment for claim amendments showing a marked up version of the amended claim in which underlines indicate insertions and brackets indicate deletions.

AMENDED CLAIMS WRITTEN IN CLEAN FORM:

69. (Amended) A method for the production of 1,2,3-trihydroxybenzene, comprising producing 1,2,3,4-tetrahydroxybenzene in accordance with claim 58 and reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

105. (New) A method for the production of 1, 2, 3, 4-tetrahydroxybenzene, comprising:

- a) incubating, in the presence of a carbon source, a first microbe comprising a recombinant DNA encoding *myo*-inositol-1-synthase, thereby forming *myo*-inositol;
- b) incubating the *myo*-inositol in the presence of a second microbe which expresses inositol dehydrogenase activity, thereby forming *myo*-2-inosose; and
- c) converting the *myo*-2-inosose to 1, 2, 3, 4-tetrahydroxybenzene by acid catalyzed dehydration.

106. (New) The method of claim 105 wherein the first microbe comprises *INO1*.

107. (New) The method of claim 106 wherein the *INO1* gene comprises a *Saccharomyces cerevisiae* *INO1*.

108. (New) The method of claim 107 wherein *INO1* comprises pAD1.88A.

109. (New) The method of claim 105 wherein the first microbe is an *Escherichia coli*.

110. (New) The method of claim 109 wherein the *Escherichia coli* is JWF1/pAD1.88A.

111. (New) The method of claim 105 wherein the second microbe is *Gluconobacter oxydans*.

112. (New) The method of claim 111 wherein the *Gluconobacter oxydans* is ATCC 621.

113. (New) The method of claim 105 wherein the second microbe comprises a recombinant DNA encoding inositol dehydrogenase.

114. (New) The method of claim 105 wherein the DNA encoding inositol dehydrogenase comprises a *Bacillus subtilis* *iolG* gene.

115. (New) The method of claim 105 wherein the carbon source comprises glucose.

116. (New) A method for the production of 1,2,3-trihydroxybenzene, comprising producing 1,2,3,4-tetrahydroxybenzene in accordance with claim 105 and reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

#### Remarks

This paper adds new claims 105-116 so that claims 58-116 are now pending in the case. No new matter is believed to be added in the amendments to the claims. Support for the new claims can be found in the specification as follows:

Claim 69 is amended to provide the correct dependency for the claim.

Claim 105 finds support in originally filed claim 1. Reference to "incubating, in the presence of a carbon source, a first microbe" finds support at least on page 7, line 25 through page 8, line 2. Reference to a microbe comprising recombinant DNA encoding *myo*-inositol-1-phosphate synthase can be found at least in claim 2 as originally filed. Reference to a microbe which expresses inositol dehydrogenase activity to produce *myo*-2-inosose can be found at least in claim 7 as originally filed and on page 5 lines 3-15.

Claims 106 and 107 find support at least in claims 3 and 4 as originally filed.

Claim 108 finds support at least in claim 5 as originally filed.

Claims 109 and 110 are supported at least by claim 6 as originally filed, on page 2, lines 20-22 and on page 6, lines 19-24.

Claims 111 and 112 are supported at least by claims 8 and 9 as originally filed.

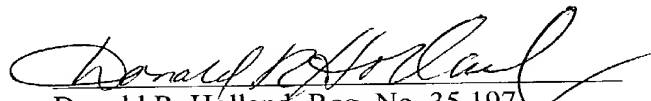
Claims 113 and 114 are supported at least by claims 10 and 11 as originally filed and on page 5, lines 4-10.

Claim 115 is supported at least by claim 12 as originally filed.

Claim 116 is supported at least by claim 13 as originally filed and on page 3, lines 27-32.

It is requested that the amendments above be entered and that the claims be examined on the merits. Should any questions arise, the Patent and Trademark Office is requested to contact the undersigned attorney.

Respectfully submitted,



Donald R. Holland, Reg. No. 35,197  
HARNESS, DICKEY & PIERCE, P.L.C.  
7700 Bonhomme  
Suite 400  
St. Louis, Missouri 63105  
(314) 726-7519



U.S. Patent and Trademark Office  
Washington, D.C. 20503  
Patent No. 7,812,111 B2  
Filed: 10/12/01  
Inventor: [illegible]  
Attorney: [illegible]

AMENDED CLAIM IN MARKED UP VERSION:

69. (Amended) A method for the production of 1,2,3-trihydroxybenzene, comprising producing 1,2,3,4-tetrahydroxybenzene in accordance with claim [1] 58 and reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

**SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1,2,3-  
TRIHYDROXYBENZENES USING MYO-INOSITOL-1-PHOSPHATE SYNTHASE  
AND MYO-INOSITOL 2-DEHYDROGENASE**

**RELATED APPLICATIONS**

The present invention is a continuation-in-part of U.S. Serial No.  
5 09/274,732, filed March 23, 1999, which is hereby expressly incorporated by  
reference.

**SPONSORSHIP**

Work on this invention was sponsored in part by the National Science  
Foundation Grant No. CHE963368. The Government may have certain rights in the  
10 invention.

**FIELD OF THE INVENTION**

The present invention is related to the production of 1,2,3,4-  
tetrahydroxybenzene and more specifically, to methods of producing 1,2,3,4-  
tetrahydroxybenzene from the bioconversion of a carbon source.

15 **BACKGROUND OF THE INVENTION**

Polyhydroxy benzenes and quinones possessing the oxygenation pattern of  
1,2,3,4-tetrahydroxybenzene **1** (Figure 1) often display biological activity.  
Aurantogliocladin **2** and fumigatin **3** (Figure 1) are antibiotics. Vischer, E.B., *J.*  
*Chem. Soc.* 815 (1953); Baker, W. et al., *J. Chem. Soc.* 820 (1953); Baker, W. et  
20 al., *J. Chem. Soc.* 670 (1941). Coenzyme Q<sub>n=10</sub> **4** (Figure 1) is an essential  
antioxidant in humans protecting low density lipoproteins from atherosclerosis-  
related oxidative modification. Ingold, K.U. et al., *PNAS (USA)* 90:45 (1993);  
Stocker, R. et al., *PNAS (USA)* 88:1646 (1991); Steinberg, D., *Circulation* 84:1420  
(1991). Dillapiole **5** (Figure 1) is a pyrethrin synergist and is responsible for the  
25 sedative effect of *Perilla frutescens* leaves. Honda, G. et al., *Chem. Pharm. Bull.*  
36:3153 (1988); Tomar, S.S. et al., *Agric. Biol. Chem.* 50:2115 (1986).

The current method of preparing 1,2,3,4-tetrahydroxybenzene uses  
pyrogallol as the synthetic starting material. Pyrogallol is converted to  
aminopyrogallol using a four-step synthesis. Aminopyrogallol is then hydrolyzed to  
30 give 1,2,3,4-tetrahydroxybenzene. Conversion of pyrogallol to 1,2,3,4-  
tetrahydroxybenzene requires the use of such reagents as phosgene, solvents such  
as pyridine and xylene, and has a nitroaromatic as a synthetic intermediate.

It would also be desirable to provide an improved method for producing  
derivatives of 1,2,3,4-tetrahydroxybenzene. Particularly, it would be desirable to

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provide a method for producing 1,2,3-trihydroxybenzene (pyrogallol). It would also be desirable if such a method were cost efficient and employed readily available materials. Currently, 1,2,3-trihydroxybenzene is obtained by thermal decarboxylation of gallic acid. However, gallic acid is isolated from natural sources such as gall nuts and tara powder and therefore is in limited supply.

It would thus be desirable to provide an improved method for producing 1,2,3,4-tetrahydroxybenzene. It would also be desirable if such a method was cost-efficient, using inexpensive starting materials. It would be further desirable if the method employed non-toxic compounds and was environmentally benign.

#### SUMMARY OF THE INVENTION

A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided. In one embodiment, the bioconversion methods of the present invention comprise the steps of microbe-catalyzed conversion of a carbon source to *myo*-2-inosose followed by acid-catalyzed dehydration of *myo*-2-inosose to produce 1,2,3,4-tetrahydroxybenzene. As shown in the synthesis scheme of Figure 2, the microbe-catalyzed conversion step of the present invention requires four enzymes. In one embodiment, the microbe-catalyzed conversion comprises the conversion of a carbon source to *myo*-inositol by a recombinant microbe and the subsequent conversion of *myo*-inositol to *myo*-2-inosose catalyzed by a second microbe. In another embodiment, the recombinant microbe is *Escherichia coli* designed to cause the conversion of glucose-6-phosphate to *myo*-inositol-1-phosphate. In yet another embodiment, the conversion of *myo*-inositol to *myo*-2-inosose is catalyzed by the microbe *Gluconobacter oxydans*. Acid-catalyzed dehydration of the resulting *myo*-2-inosose yields 1,2,3,4-tetrahydroxybenzene.

The biocatalytic synthesis of 1,2,3,4-tetrahydroxybenzene provided herein is environmentally benign, economically attractive, and utilizes abundant renewable sources as a starting material.

Methods are also provided for the production of derivatives of 1,2,3,4-tetrahydroxybenzene, particularly Coenzyme Q 1,2,3-trihydroxybenzene (pyrogallol). In one embodiment 1,2,3-trihydroxybenzene is produced by reduction of 1,2,3,4-tetrahydroxybenzene. In a preferred embodiment, the reduction is achieved by catalytic hydrogenation followed by hydrolysis.

Additional objects, advantages, and features of the present invention will become apparent from the following description and appended claims, taken in conjunction with the accompanying drawings.

### BRIEF DESCRIPTION OF THE DRAWINGS

5       The various advantages of the present invention will become apparent to one skilled in the art by reading the following specification and subjoined claims and by referencing the following drawings in which:

Figure 1 is an illustration showing the structures of products that can be derived from 1,2,3,4-tetrahydroxybenzene;

10       Figure 2 is a schematic illustrating the bioengineered synthesis scheme of the present invention for producing 1,2,3,4-tetrahydroxybenzene;

Figure 3 is a graph showing the production of *myo*-inositol in comparison to cell dry weight;

15       Figure 4 is a schematic illustrating the conventional synthetic scheme for synthesizing 1,2,3,4-tetrahydroxybenzene; and

Figure 5 is a schematic illustrating the synthesis scheme for converting 1,2,3,4-tetrahydroxybenzene to Coenzyme Q.

### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20       A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided herein. Methods of producing 1,2,3,4-tetrahydroxybenzene from a carbon source based on the synthesis scheme are also provided. In one embodiment, a method is provided wherein the carbon source is converted to *myo*-inositol by a recombinant microbe, the *myo*-inositol is further converted to *myo*-2-inosose by a second microbe,  
25       followed by acid-catalyzed dehydration of *myo*-2-inosose to produce 1,2,3,4-tetrahydroxybenzene.

Novel methods are also provided for the production of derivatives of 1,2,3,4-tetrahydroxybenzene, particularly 1,2,3-trihydroxybenzene (pyrogallol). In one embodiment 1,2,3-trihydroxybenzene is produced by reduction of 1,2,3,4-tetrahydroxybenzene. In a preferred embodiment, the reduction is achieved by  
30       catalytic hydrogenation of 1,2,3,4-tetrahydroxybenzene followed by acid catalyzed hydrolysis to yield 1,2,3-trihydroxybenzene. In a more preferred embodiment, the hydrogenation is catalyzed by Rh/Al<sub>2</sub>O<sub>3</sub>. These methods take advantage of inexpensive and abundant carbon sources as starting materials to produce 1,2,3,4-tetrahydroxybenzene which can then be converted to 1,2,3-trihydroxybenzene.  
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Although microbe-catalyzed conversion of a carbon source to *myo*-inositol by a recombinant microbe followed by conversion of the *myo*-inositol to *myo*-2-inosose by a second microbe is described in detail herein, in an alternative embodiment, a single recombinant microbe is employed to convert a carbon source  
5 directly to *myo*-2-inosose which is then converted to 1,2,3,4-tetrahydroxybenzene by an acid-catalyzed dehydration. This single-microbe conversion may be carried out by any type of microbe sufficiently engineered to produce the desired outcome.

In another alternative embodiment, a recombinant microbe catalyzes the conversion of a carbon source to D-2,3-diketo-4-deoxy-*epi*-inositol which is  
10 subsequently converted to 1,2,3,4-tetrahydroxybenzene by an acid-catalyzed dehydration. D-2,3-Diketo-4-deoxy-*epi*-inositol is an intermediate in the microbial catabolism of *myo*-inositol as well as a likely intermediate in the acid-catalyzed conversion of *myo*-2-inosose into 1,2,3,4-tetrahydroxybenzene.

The bioconversion methods of the present invention are carried out under  
15 conditions of time, temperature, pH, nutrient type and concentration, aeration conditions and glucose concentrations, to provide maximal conversion of the carbon source to 1,2,3,4-tetrahydroxybenzene. As described in detail in Specific Example 1, in a preferred embodiment, a fed-batch fermentor is used to convert the carbon source to *myo*-inositol, followed by isolation of *myo*-inositol, *e.g.*, deionization and  
20 decolorization of the fermentation broth and precipitation by the addition of an organic solvent. The isolated *myo*-inositol is then converted to *myo*-inositol to *myo*-2-inosose followed by isolation of the *myo*-2-inosose, *e.g.*, precipitation of the *myo*-2-inosose from the culture broth. The fed-batch fermentor process and the precipitation methods are also known to those skilled in the art.

25 As used herein, the phrase "carbon source" is meant to include biomass-derived carbon sources including, but not limited to, xylose, arabinose, glycerol, glucose and the intermediates (*e.g.*, dicarboxylic acids) in the Krebs cycle, either alone or in combination. In a preferred embodiment, the carbon source is glucose.

In one embodiment, a recombinant *E. coli* microbe is employed in the  
30 methods of the present invention. In a preferred embodiment, the *E. coli* comprises a non-functional *serA* locus. This recombinant *E. coli*, designated, JWF1, may further comprise a plasmid carrying an *INO1* gene insert and a *serA* gene insert. The *INO1* gene encodes *myo*-inositol-1-phosphate synthase which converts glucose-6-phosphate to *myo*-inositol-1-phosphate. In a preferred embodiment, the  
35 *INO1* gene is from *Saccharomyces cerevisiae*. Overexpression of *myo*-inositol-1-

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phosphate synthase will increase carbon flow into the *myo*-inositol pathway. This recombinant microbe is capable of converting glucose to *myo*-inositol.

In another embodiment, the *myo*-inositol produced by the first recombinant microbe, is converted to *myo*-2-inosose by a second microbe. This second microbe  
5 can either be a recombinant microbe or a naturally occurring microbe. A recombinant microbe comprises a plasmid carrying the *ioIG* gene insert. The *ioIG* gene insert encodes the enzyme inositol dehydrogenase, which catalyzes the conversion of *myo*-inositol to *myo*-2-inosose. Alternatively, the *ioIG* gene insert is inserted directly into the genome of the recombinant microbe. Preferably, the *ioIG*  
10 gene is isolated from *Bacillus subtilis*. In a preferred embodiment, the second microbe is a naturally occurring microbe that express inositol dehydrogenase activity. Examples of such microbes include, but are not limited to, *Bacillus subtilis* and *Gluconobacter oxydans*. In a preferred embodiment, the second microbe is *G. oxydans*, which converts *myo*-inositol to *myo*-2-inosose without loss of the *myo*-2-  
15 inosose product to further catabolism.

In a preferred embodiment, the recombinant *E. coli* comprises plasmid pAD1.88A carrying an *INO1* gene insert and a *serA* gene insert. As described above, the *INO1* gene insert encodes *myo*-inositol-1-phosphate synthase which converts glucose-6-phosphate to *myo*-inositol-1-phosphate, thus increasing the  
20 carbon flow into the *myo*-inositol pathway. Due to a mutation in the *E. coli* genomic *serA* locus required for L-serine biosynthesis, growth in minimal salts medium and plasmid maintenance follows from expression of plasmid-localized *serA*. The *serA* insert thus allows microbial growth in minimal salts medium, distinguishing the microbes containing the plasmid from non-plasmid containing  
25 microbes.

In an alternative embodiment, the recombinant *E. coli* comprises a plasmid carrying an *INO1* gene insert, an *ioIG* gene insert and a *serA* gene insert. As described above, the *ioIG* gene insert encodes inositol dehydrogenase which catalyzes the conversion of *myo*-inositol to *myo*-2-inosose. In a preferred  
30 embodiment, the plasmid also carries the gene insert for inositol monophosphatase. While not wishing to be bound by theory, hydrolysis of *myo*-inositol-1-phosphate to produce *myo*-inositol can occur in the cytosol or periplasm. If a cytoplasmic phosphatase hydrolyzes *myo*-inositol-1-phosphate, plasmid-localized *INO1* and *ioIG* will lead to *myo*-2-inosose synthesis. Periplasmic phosphatase activity would result  
35 in periplasmic production of *myo*-inositol while inositol dehydrogenase expression

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is localized in the cytoplasm. Transport of *myo*-inositol from the periplasm into the cytoplasm is unlikely in *E. coli* given that this microbe does not catabolize *myo*-inositol. To correct for periplasmic phosphatase activity, plasmid-localization of genes encoding mammalian inositol monophosphatase, which have been cloned, sequenced, and successfully expressed in *E. coli*, would be desirable. Diehl, R.E. et al., *J. Biol. Chem.* 265:5946 (1990); McAllister, G. et al., *Biochem. J.* 284:749 (1992). Because of the specificity of this enzyme for *myo*-inositol-1-phosphate, a molecule which is not a normal metabolite in *E. coli*, cytoplasmic expression of the cDNA encoding inositol monophosphatase in *E. coli* should not be problematic. An *E. coli* comprising a plasmid carrying both the *INO1* gene insert and the *iolG* gene insert and the gene for inositol monophosphatase can convert glucose directly to *myo*-2-inosose. The *myo*-2-inosose can then be converted to 1,2,3,4-tetrahydroxybenzene by an acid-catalyzed dehydration.

In another embodiment, the recombinant *E. coli*, designated JWF1, comprises a plasmid carrying an *INO1* gene insert, an *iolG* gene insert, and a *serA* gene insert. This recombinant microbe is capable of converting glucose to *myo*-2-inosose. In a preferred embodiment, the recombinant *E. coli* comprises plasmid pAD2.28A carrying an *INO1* gene insert, an *iolG* gene insert and a *serA* gene insert. Examples of these recombinant microbes, *E. coli* JWF1/pAD1.88A and JWF1/pAD2.28A, are described in Specific Examples 1 and 4, respectively, and have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20582, under the terms of the Budapest Treaty, and accorded the ATCC designation numbers 207153 and 207154, respectively. The deposit will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of a patent, whichever is longer, and will be replaced if the deposit becomes depleted or non-viable during that period. Samples of the deposit will become available to the public and all restrictions imposed on access to the deposit will be removed upon grant of a patent on this application.

In yet another embodiment, a recombinant *E. coli* is employed to convert glucose to D-2,3-diketo-4-deoxy-*epi*-inositol. Such a recombinant *E. coli* comprises a plasmid carrying the *INO1* gene insert, the *iolG* gene insert and the gene insert encoding for the enzyme, *myo*-2-inosose dehydratase. The plasmid may further comprise the gene insert for inositol monophosphatase. *Myo*-2-inosose dehydratase catalyzes the conversion of *myo*-2-inosose to D-2,3-diketo-4-deoxy-*epi*-inositol

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which can undergo an acid-catalyzed dehydration to yield 1,2,3,4-tetrahydroxybenzene. While not wishing to be bound by theory, D-2,3-diketo-4-deoxy-*epi*-inositol is likely the first intermediate in the acid-catalyzed conversion of *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene. Acid-catalyzed aromatization of  
5 D-2,3-diketo-4-deoxy-*epi*-inositol would eliminate a step and may lead to higher yields of 1,2,3,4-tetrahydroxybenzene as compared to the acid-catalyzed dehydration of *myo*-2-inosose.

It will be appreciated that the *INO1* gene, *iolG* gene, *serA* gene and the genes encoding inositol dehydrogenase, inositol monophosphatase and/or *myo*-2-  
10 inosose dehydratase, can be inserted directly into the *E. coli* genome. Such a recombinant *E. coli* would not require a plasmid to produce significant amounts of *myo*-inositol, *myo*-2-inosose or D-2,3-diketo-4-deoxy-*epi*-inositol.

Although *E. coli* is specifically described herein as the microbe for carrying out the methods of the present invention, it will be appreciated that any  
15 microorganism such as the common types cited in the literature and known to those skilled in the art, may be employed, provided the microorganism can be altered to effect the desired conversion (*e.g.*, carbon source to *myo*-inositol, carbon source to *myo*-2-inosose, carbon source to D-2,3-diketo-4-deoxy-*epi*-inositol, *myo*-inositol to *myo*-2-inosose, etc.). Thus it is envisaged that many types of fungi, bacteria and  
20 yeasts will work in the methods of the present invention. Such microorganisms may be developed, for example, through selection, mutation, and/or genetic transformation processes with the characteristic and necessary capability of converting one constituent of the synthesis scheme of the present invention to another. Methods for such development are well known to the skilled practitioner.

25 In order to carry out the bioconversion methods of the present invention, a solution containing a carbon source is contacted with the recombinant or wild-type microbe to form a bioconversion mixture which is maintained under appropriate conditions to promote the conversion of the carbon source to the desired constituent, *e.g.*, *myo*-inositol or *myo*-2-inosose. In a preferred embodiment, the  
30 bioconversion mixture is maintained at a temperature of about 30°C to about 37°C and a pH of about 6.5 to about 7.5. It is preferred that the bioconversion mixture also contain other substances necessary to promote viability of the microbes such  
as mineral salts, buffers, cofactors, nutrient substances and the like. The more  
general requirements for the maintenance of viability of microorganisms are well  
35 known and specific requirements for maintaining the viability of specific organisms



are also well known as documented in the literature, or are otherwise easily determined by those skilled in the art.

In another embodiment, the *myo*-inositol produced in the fermentation broth of the first bioconversion with the first microbe can be isolated before being utilized in the second bioconversion. The isolation can be a total isolation to provide pure *myo*-inositol as described in Specific Example 1. The isolation can also be a partial isolation where the fermentation broth is deproteinized and decolorized before use in the second bioconversion. Such deproteinization and decolorization are well known to those skilled in the art. Further purification of the *myo*-inositol can be obtained by concentration of the deproteinized/decolorized fermentation broth to a volume where the *myo*-inositol concentration is greater than 50 g/L. At concentrations greater than 50 g/L, the *myo*-inositol can be precipitated out by the addition of methanol. By way of non-limiting example, *myo*-inositol can be isolated from the fermentation broth by the following four steps: ultrafiltration to remove cells and proteins; decolorization by activated charcoal; concentration of the fermentation broth to give a *myo*-inositol concentration greater than 50 g/L; and precipitation of *myo*-inositol by the addition of methanol. The *myo*-inositol precipitate can be washed, resuspended in buffer or water, and used in the second bioconversion to *myo*-2-inosose.

Novel methods for converting 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene are also provided. In one embodiment, a method is provided wherein 1,2,3,4-tetrahydroxybenzene is reduced to 1,2,3-trihydroxybenzene.

In one embodiment, the 1,2,3,4-tetrahydroxybenzene is converted to 1,2,3-trihydroxybenzene by hydrogenation in the presence of a catalyst followed by acid catalyzed hydrolysis. In a preferred embodiment, the catalyst is Rh/Al<sub>2</sub>O<sub>3</sub>. The amount of catalyst required and the conditions required for hydrogenation (e.g., pressure, time) are well known to the skilled practitioner.

In another embodiment, the hydrogenation reaction is carried out in an aqueous 1,2,3,4-tetrahydroxybenzene solution. In a preferred embodiment, the aqueous 1,2,3,4-tetrahydroxybenzene solution will be free of any compounds known to quench the hydrogenolysis catalyst. In a more preferred embodiment, the aqueous 1,2,3,4-tetrahydroxybenzene solution is comprised of isolated 1,2,3,4-tetrahydroxybenzene and water.

In order to more fully demonstrate the advantages arising from the present invention, the following examples are set forth. It is to be understood that the

following is by way of example only and is not intended as a limitation on the scope of the invention.

### SPECIFIC EXAMPLE 1

#### Synthesis Of 1,2,3,4-Tetrahydroxybenzene From Glucose

##### 5 I. Results

A synthetic route (Figure 2) has now been elaborated which provides convenient access to 1,2,3,4-tetrahydroxybenzene via *myo*-inositol intermediacy. The general utility of this route is demonstrated by a concise synthesis of coenzyme Q<sub>n=3</sub> **4**. While the shikimate pathway and polyketide biosynthesis have traditionally  
10 provided biocatalytic access to aromatic chemicals, syntheses of 1,2,3,4-tetrahydroxybenzene **1** and coenzyme Q<sub>n=3</sub> **4** are distinguished by the recruitment of *myo*-inositol biosynthesis.

Synthesis of *myo*-inositol by *E. coli* JWF1/pAD1.88A begins with D-glucose uptake and conversion to D-glucose-6-phosphate catalyzed by the *E. coli*  
15 phosphotransferase system (Postma, P.W. et al., In *Escherichia coli and Salmonella*, 2nd ed., Neidhardt, F.C. et al., Eds., ASM: Washington, Vol. 1, p. 1149 (1996)) where phosphoenolpyruvate is the source of the transferred phosphoryl group. D-Glucose-6-phosphate then undergoes cyclization to *myo*-inositol 1-phosphate catalyzed by *myo*-inositol-1-phosphate synthase. This enzyme activity, which  
20 results from expression of the *Saccharomyces cerevisiae* *INO1* gene (Dean-Johnson, M. et al., *J. Biol. Chem.* 264:1274 (1989)) on plasmid pAD1.88A, varied significantly (0.022, 0.043, 0.018, and 0.009  $\mu\text{mol}/\text{min}/\text{mg}$  at 18 h, 30 h, 42 h, and 54 h, respectively) over the course of the fermentation.

*E. coli* JWF1/pAD1.88A synthesized 21 g/L *myo*-inositol (solid bars, Figure  
25 3) and 4 g/L *myo*-inositol-1-phosphate (open bars, Figure 3) in 11% combined yield (mol/mol) from D-glucose under fed-batch fermentor conditions. Both *myo*-inositol and *myo*-inositol-1-phosphate accumulated in the culture supernatant. In eucaryotes, hydrolysis of *myo*-inositol-1-phosphate to *myo*-inositol is catalyzed by the enzyme inositol monophosphatase. McAllister, G. et al., *Biochem. J.* 284:749  
30 (1992). Phosphoester hydrolysis was fortuitously catalyzed in *E. coli* JWF1/pAD1.88A by unidentified cytosolic or periplasmic phosphatase activity.

Oxidation of *myo*-inositol to *myo*-2-inosose, the next step in the conversion of D-glucose into 1,2,3,4-tetrahydroxybenzene **1**, is the first catabolic step when *myo*-inositol is used as a sole source of carbon for growth and metabolism by  
35 microbes such as *Bacillus subtilis*. Yoshida, K.-I. et al., *J. Bacteriol.* 179:4591

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(1997). *myo*-Inositol can also be oxidized by *Gluconobacter oxydans* without loss of product *myo*-2-inosose to catabolism. Posternak, T., *Bioch. Prep.* 2:57 (1952). Accordingly, incubation of *G. oxydans* ATCC 621 in medium containing microbe-synthesized *myo*-inositol led to the formation of *myo*-2-inosose (Scheme 1) in 95%  
5 isolated yield.

Inososes have been thought to be stable under acidic conditions and reactive under basic conditions with reported aromatizations resulting from successive  $\beta$ -eliminations being dominated by formation of 1,2,3,5-tetrahydroxybenzene. Posternak, T., *The Cyclitols*, Holden-Day: San Francisco, Chap. 8 (1965); Angyal, S.J. et al., *Carbohydr. Res.* 76:121 (1979). However, it was observed that *myo*-2-inosose was reactive under acidic conditions with no apparent formation of 1,2,3,5-tetrahydroxybenzene. Refluxing *G. oxydans*-produced *myo*-2-inosose for 9 h in degassed, aqueous 0.5 M  $H_2SO_4$  under argon cleanly afforded 1,2,3,4-tetrahydroxybenzene in 66% isolated yield.

## 15 II. Materials And Methods

**General.**  $^1H$  NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts for  $^1H$  NMR spectra are reported (in parts per million) relative to internal tetramethylsilane ( $Me_4Si$ ,  $\delta = 0.0$  ppm) with  $CDCl_3$  as solvent, to sodium 3-(trimethylsilyl)propionate-2,2,3,3- $d_4$  (TSP,  $\delta = 0.0$  ppm) when  $D_2O$  was the  
20 solvent, and to acetone ( $CHD_2COCD_3$ ,  $\delta = 2.04$  ppm) with  $d_6$ -acetone.  $^{13}C$  NMR spectra were recorded at 75 MHz. Chemical shifts for  $^{13}C$  NMR spectra are reported (in parts per million) relative to  $CDCl_3$  ( $\delta = 77.0$  ppm), relative to  $CD_3COCD_3$  ( $\delta = 29.8$  ppm), and relative to internal  $CH_3OH$  ( $\delta = 49.0$  ppm) or internal  $CH_3CN$  ( $\delta = 1.4$  ppm) in  $D_2O$ . FAB mass spectra were performed by  
25 University of South Carolina (Columbia, SC). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

Radial chromatography was carried out with a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60  $PF_{254}$  containing gypsum  
30 (E. Merck). Silica gel 60 (40 - 63  $\mu m$  E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, E. Merck or Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) followed by heating. Dimethylformamide, N-methylformanilide and acetone were dried and stored over activated Linde 4Å  
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molecular sieves under Ar. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. *n*-Hexanes and TMEDA were distilled from sodium under Ar and stored over activated Linde 4Å molecular sieves under Ar. Organic solutions of products were dried over MgSO<sub>4</sub>.

5 For <sup>1</sup>H NMR quantitation of solute concentrations during microbial synthesis of *myo*-inositol and *myo*-2-inosose, solutions were concentrated to dryness under reduced pressure, concentrated to dryness one additional time from D<sub>2</sub>O, and then redissolved in D<sub>2</sub>O containing a known concentration of TSP purchased from Lancaster Synthesis Inc. Concentrations were determined by comparison of  
10 integrals corresponding to each compound with the integral corresponding to TSP ( $\delta$  = 0.00 ppm) in the <sup>1</sup>H NMR. Protein concentrations were determined using the Bradford dye-binding procedure (Bradford, M.M., *Anal. Biochem.* 72:248 (1979)) by comparison with a standard curve prepared with bovine serum albumin. Protein assay solution was purchased from Bio-Rad. *E. coli* DH5 $\alpha$  is available from Gibco  
15 BRL.

**Culture Medium.** All culture solutions were prepared in distilled, deionized water. LB medium (1 L) contained Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g). M9 salts (1 L) contained Na<sub>2</sub>HPO<sub>4</sub> (6 g), KH<sub>2</sub>PO<sub>4</sub> (3 g), NaCl (0.5 g) and NH<sub>4</sub>Cl (1 g). M9 minimal medium (1 L) consisted of 1 L of M9 salts  
20 containing D-glucose (10 g), MgSO<sub>4</sub> (0.12 g), and thiamine hydrochloride (0.001 g). Ampicillin was added (0.05 g/L) where indicated. Solutions of M9 salts, MgSO<sub>4</sub>, and glucose were autoclaved individually and then mixed. Ampicillin was sterilized through a 0.22  $\mu$ m membrane. Solid medium was prepared by addition of 1.5% (w/v) Difco agar to medium.

25 Fermentation medium (1 L) contained K<sub>2</sub>HPO<sub>4</sub> (7.5 g), ammonium iron(III) citrate (0.3 g), citric acid monohydrate (2.1 g), and concentrated H<sub>2</sub>SO<sub>4</sub> (1.2 mL). The fermentation medium was adjusted to pH 7.0 by addition of concentrated NH<sub>4</sub>OH before autoclaving. The following supplements were added immediately prior to initiation of the fermentation: D-glucose (20 g), MgSO<sub>4</sub> (0.24 g), and trace  
30 minerals including (NH<sub>4</sub>)<sub>6</sub>(Mo<sub>7</sub>O<sub>24</sub>)·4H<sub>2</sub>O (0.0037 g), ZnSO<sub>4</sub>·7H<sub>2</sub>O (0.0029 g), H<sub>3</sub>BO<sub>3</sub> (0.0247 g), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.0025 g), and MnCl<sub>2</sub>·4H<sub>2</sub>O (0.0158 g). D-Glucose and MgSO<sub>4</sub> were autoclaved separately while trace minerals were sterilized through 0.22  $\mu$ m membranes prior to addition to the medium.

**Genetic Manipulations.** Standard procedures were used for the construction,  
35 purification, and analysis of plasmid DNA. Sambrook, J. et al., *Molecular Cloning*

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*A Laboratory Manual*, Cold Spring Harbor Laboratory Press: New York (1989). *E. coli* DH5 $\alpha$  served as the host strain for all plasmid constructions. The *INO1* open reading frame was amplified from pJH318 (Hirsch, J.P. et al., *Mol. Cell. Biol.* 6:3320 (1986)) using PCR. Inclusion of *EcoRI* recognition sequences facilitated localization of *INO1* into the *EcoRI* site in pJF118EH (Furste, J.P. et al., *Gene* 48:119 (1986)) to afford pAD1.45A. Transcription of *INO1* in pAD1.45A utilized the vector-encoded *tac* promoter ( $P_{tac}$ ) which was regulated by vector-encoded expression of *lac*<sup>r</sup>. Digestion of pD2625 with *DraI* and *EcoRV* liberated a 1.9 kb *serA* fragment that was subsequently ligated into the *SmaI* site of pAD1.45A to provide pAD1.88A.

*E. coli* JWF1 was prepared by homologous recombination of a non-functional *serA* gene into *E. coli* RB791(W3110 *lac*<sup>r</sup>). Localization of the 1.9 kb *serA* fragment obtained from pD2625 into pMAK705 (Hamilton, C.M. et al., *J. Bacteriol.* 171:4617 (1989)) provided pLZ1.68A. Linearization of pLZ1.68A at the unique *Bam*HI site internal to *serA* followed by treatment with Klenow fragment and dNTP's and relegation afforded pLZ1.71A. Homologous recombination (Ohta, K. et al., *Appl. Environ. Microbiol.* 57:893 (1991)) of the resulting non-functional *serA* locus of pLZ1.71A into RB791 afforded JWF1.

***myo-Inositol-1-phosphate synthase activity.*** Partial purification of cellular lysate was required to quantify *myo*-inositol-1-phosphate synthase activity over background cellular phosphatase activity. Cells were collected from 30 mL of fermentation broth by centrifugation at 2000g for 6 min at 4 °C. Cells were resuspended in 10 mL of resuspension buffer consisting of Tris·HCl (20 mM), pH 7.4, NH<sub>4</sub>Cl (10 mM), 2-mercaptoethanol ( $\beta$ -ME, 10 mM), phenylmethylsulphonyl fluoride (PMSF, 2 mM), and EDTA (1 mM). Resuspended cells were frozen at -80 °C for up to 4 days until purification was carried out.

Thawed cells were lysed by two passages through a French press at 2000 psi. Cellular debris was removed by centrifugation at 30000g for 30 min at 4 °C. Clarified cellular lysate containing approximately 200 mg of protein was loaded onto a DEAE cellulose column (5 x 25 cm) at 4 °C. The column was eluted with a step gradient of NH<sub>4</sub>Cl in the following buffer (Buffer A): Tris HCl (20 mM), pH 7.4,  $\beta$ -ME (10 mM), PMSF (1 mM), and EDTA (1 mM). The step gradient consisted of 20 mL of Buffer A with NH<sub>4</sub>Cl (10 mM), 45 mL of Buffer A with NH<sub>4</sub>Cl (90 mM), and 100 mL of Buffer A with NH<sub>4</sub>Cl (150 mM). Fractions (9 mL) were collected throughout the step gradient. Fractions 10-18 were collected and concentrated to

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less than 5 mL using an Amicon Ultrafiltration Stirred Cell equipped with a PM10 membrane. Concentrated protein (1.5-2.0 mg) was used to measure *myo*-inositol-1-phosphate synthase activity. The *myo*-inositol-1-phosphate synthase activity was measured as previously reported (Migaud, M.E. et al., *J. Am. Chem. Soc.* 118:495  
5 (1996)) except that the assay solution contained Tris HCl (20 mM), pH 7.4, NH<sub>4</sub>Cl (2 mM), and DTT (0.2 mM).

***myo-Inositol.*** Fermentations employed a 2.0 L working capacity B. Braun MD2 culture vessel. Utilities were supplied by a B. Braun Biostat MD controlled by a Dell Optiplex Gs<sup>+</sup> 5166 personal computer equipped with B. Braun MFCS/Win  
10 software. Temperature, pH, and glucose feeding were controlled with PID control loops. Temperature was maintained at 33 °C. pH was maintained at 7.0 by addition of concentrated NH<sub>4</sub>OH or 2 N H<sub>2</sub>SO<sub>4</sub>. Dissolved oxygen (D.O.) was measured using a Mettler-Toledo 12 mm sterilizable O<sub>2</sub> sensor fitted with an Ingold A-type O<sub>2</sub> permeable membrane. D.O. was maintained at 10% air saturation.  
15 Antifoam (Sigma 204) was added manually as needed.

Inoculants were started by introduction of a single colony of JWF1/pAD1.88A into 100 mL M9 medium containing ampicillin. The culture was grown at 37 °C with agitation at 250 rpm for 15 h and then transferred to the fermentation vessel. The initial glucose concentration in the fermentation medium  
20 was 20 g/L. Three staged methods were used to maintain D.O. levels at 10% air saturation during each fermentor run. With the airflow at an initial setting of 0.06 L/L/min, D. O. concentration was maintained by increasing impeller speed from its initial set point of 50 rpm to its preset maximum of 940 rpm. Approximately 7 h was required for the impeller speed to increase to 940 rpm. With the impeller  
25 constant at 940 rpm, the mass flow controller then maintained D.O. levels by increasing the airflow rate from 0.06 L/L/min to its preset maximum of 1.0 L/L/min over approximately 1.5 h. At constant impeller speed and constant airflow rate, D.O. levels were maintained at 10% saturation for the remainder of the fermentation by oxygen sensor-controlled glucose feeding. At the beginning of this  
30 stage, D. O. levels fell below 10% air saturation due to residual initial glucose in the medium. This lasted for approximately 50 min before glucose (60% w/v) feeding started. The PID control parameters were set to 0.0 (off) for the derivative control ( $\tau_D$ ) and 999.9 s (minimum control action) for integral control ( $\tau_I$ ).  $X_p$  was set to 950% to achieve a  $K_c$  of 0.1.

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Samples (6 mL) of fermentation broth were taken at 6 h intervals starting at 12 h. Isopropyl- $\beta$ -D-thiogalactopyranoside (4.8 mg) was added when both the impeller speed and airflow had reached the maximum settings, and again at 12 h and every 6 h thereafter. Cell densities were determined by dilution of fermentation  
5 broth with water (1:100) followed by measurement of absorption at 600 nm ( $OD_{600}$ ). Dry cell weight (g/L) was obtained using a conversion coefficient of 0.43 g/L/ $OD_{600}$ . Fermentation broth was centrifuged to remove cells. Solute concentrations in cell-free broth were determined by  $^1H$  NMR. Fermentation broth (30 mL) was removed at designated times for assay of *myo*-inositol-1-phosphate  
10 synthase activity. The final concentration of *myo*-inositol at 54 h was 20.9 g/L synthesized in 8.7% yield (mol/mol) from glucose.

The fermentation broth (950-1200 mL) was centrifuged at 18000g for 35 min at 4 °C and the cells discarded. The resulting supernatant was acidified to pH 2.0 with concentrated  $H_2SO_4$  and then centrifuged at 18000g for 20 min to remove  
15 precipitated proteins. The clear yellow supernatant was neutralized with concentrated  $NH_4OH$ . The solution was decolorized with Darco KB-B activated carbon (10 g/L) for 4 h with agitation at 50 rpm and subsequently filtered through Whatman 2 filter paper. The filtered material was washed with an additional 200 mL of water.

20 The combined filtrates were applied to a column of AG1-x8 (acetate form, 5 cm x 20 cm) at 4 °C and eluted with 1 L  $H_2O$ . The entire eluent (approximately 2.3 L) was then run through a column of Dowex 50 ( $H^+$  form, 5 cm x 20 cm) at 4 °C and eluted with 500 mL  $H_2O$ . The resulting solution (approximately 2.8 L) was concentrated to 200 mL by boiling and then concentrated to dryness under reduced  
25 pressure. The resulting powder was dissolved in a minimal volume of  $H_2O$ , diluted with 6 volumes of MeOH, and stored at 4 °C to crystallize. Crystals were collected after a few days, washed with MeOH, allowed to air dry overnight, and dried under vacuum to yield white crystals (78 % recovery based on inositol quantified in crude fermentation broth).  $^1H$  NMR ( $D_2O$ )  $\delta$  4.06 (dd,  $J=3, 3$  Hz, 1 H), 3.61 (dd,  $J=10,$   
30 9 Hz, 2 H), 3.53 (ddd,  $J=10, 3, 1$  Hz, 2 H), 3.28 (ddd,  $J=9, 9, 1$  Hz, 1 H).  $^{13}C$  NMR ( $D_2O$ )  $\delta$  45.3, 43.4, 43.2, 42.1.

***myo-2-Inosose.*** Angyal, S.J. et al. *Carbohydr. Res.* 76:121 (1979); Posternak, T., *Biochem. Prep.* 2:57 (1952). A solution containing sorbitol (1.0 g) and yeast extract (0.05 g) in 10 mL distilled, deionized water was autoclaved for  
35 25 min and cooled to room temperature. After inoculation with *Gluconobacter*

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*oxydans* ATCC 621 the culture was incubated in an orbital shaker at 200 rpm for 24 h at 30 °C. This *G. oxydans* culture was subsequently added to a second sterile solution containing *myo*-inositol (12.0 g, 66.7 mmol), D-sorbitol (0.4 g), and yeast extract (2.0 g) in 400 mL distilled, deionized water. After incubation in an orbital shaker at 200 rpm for 48 h at 30 °C, cells were removed by centrifugation. The resulting culture supernatant was concentrated to 75 mL, MeOH (400 mL) added, and the solution maintained at -20 °C for 12 h. Precipitate which formed was filtered, washed with MeOH, and dried to afford *myo*-2-inosose as a white powder (8.17 g, 69%). A second crop of *myo*-2-inosose (3.09 g, 26%) was obtained after maintaining the filtrate at -20 °C for an additional 12 h. mp 188 -192 °C. <sup>1</sup>H NMR (D<sub>2</sub>O): δ 4.25 (d, *J* = 10 Hz, 2 H), 3.66 (dd, *J* = 9, 9 Hz), 3.26 (m, 2 H). <sup>13</sup>C NMR (D<sub>2</sub>O): δ 206.0, 94.3, 76.2, 74.5, 74.1, 74.0, 73.3, 73.2.

**1,2,3,4-Tetrahydroxybenzene 1.** A solution of *myo*-2-inosose (11.0 g, 61.2 mmol) in 310 mL of degassed 0.5 M H<sub>2</sub>SO<sub>4</sub> was refluxed under Ar. After 9 h, the solution was cooled to 4 °C and then adjusted to pH 4 by addition of saturated aqueous NaHCO<sub>3</sub>. Concentration of the reaction solution to 100 mL was followed by continuous liquid-liquid extraction for 18 h using *t*-butyl methyl ether (500 mL). Upon concentration of the organic layer to 100 mL, a precipitate formed which was filtered, washed with cold hexanes, and dried to afford **1** (4.72 g, 54%) as a tan powder. Addition of hexanes (300 mL) to the filtrate followed by filtering, washing, and drying of the resulting precipitate afforded additional **1** (1.08 g, 12%). mp 162 - 164 °C. <sup>1</sup>H NMR (*d*<sub>6</sub>-acetone): δ 7.24 (s, 4 H), 6.20 (s, 2 H). <sup>13</sup>C NMR (*d*<sub>6</sub>-acetone): δ 139.7, 134.7, 106.2. Anal. Calcd for C<sub>6</sub>H<sub>6</sub>O<sub>4</sub>: C, 50.71; H, 4.23. Found: C, 50.63; H, 4.32. HRMS (FAB) calcd for C<sub>6</sub>H<sub>6</sub>O<sub>4</sub> (M + H<sup>+</sup>): 142.0266. Found: 142.0268.

## SPECIFIC EXAMPLE 2

### Chemical Synthesis Of 1,2,3,4-Tetrahydroxybenzene

#### I. Results

Conversion of D-glucose into 1,2,3,4-tetrahydroxybenzene **1** is a three step synthesis. 1,2,3,4-Tetrahydroxybenzene **1** has historically been obtained from pyrogallol **6** by a longer route (Figure 4) involving synthesis and subsequent hydrolysis of aminopyrogallol **7**. Leston, G., In *Kirk-Othmer Encyclopedia of Chemical Technology*: Fourth Ed., Kroschwitz, J.I. et al. Ed., Wiley: New York, Vol. 19, p. 778 (1996); Einhorn, A. et al., *Ber.* 37:110 (1904). Due to the tedious nature of this synthesis (Einhorn, A. et al., *Ber.* 37:110 (1904)), two alternate



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routes (Figure 4) were developed to obtain authentic samples of 1,2,3,4-tetrahydroxybenzene **1**. Low-yielding, direct hydroxylation of protected pyrogallol **8** or higher-yielding, indirect oxidation via formyl **10** intermediacy yielded, respectively, quinone **9** and phenol **11**. Hydrogenation of **9** and **11** afforded products which were identical to 1,2,3,4-tetrahydroxybenzene **1** synthesized (Figure 2) from D-glucose.

## II. Materials And Methods

**General.**  $^1\text{H}$  NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts for  $^1\text{H}$  NMR spectra are reported (in parts per million) relative to internal tetramethylsilane ( $\text{Me}_4\text{Si}$ ,  $\delta = 0.0$  ppm) with  $\text{CDCl}_3$  as solvent, to sodium 3-(trimethylsilyl)propionate-2,2,3,3- $\text{d}_4$  (TSP,  $\delta = 0.0$  ppm) when  $\text{D}_2\text{O}$  was the solvent, and to acetone ( $\text{CHD}_2\text{COCD}_3$ ,  $\delta = 2.04$  ppm) with  $d_6$ -acetone.  $^{13}\text{C}$  NMR spectra were recorded at 75 MHz. Chemical shifts for  $^{13}\text{C}$  NMR spectra are reported (in parts per million) relative to  $\text{CDCl}_3$  ( $\delta = 77.0$  ppm), relative to  $\text{CD}_3\text{COCD}_3$  ( $\delta = 29.8$  ppm), and relative to internal  $\text{CH}_3\text{OH}$  ( $\delta = 49.0$  ppm) or internal  $\text{CH}_3\text{CN}$  ( $\delta = 1.4$  ppm) in  $\text{D}_2\text{O}$ . FAB mass spectra were performed by University of South Carolina (Columbia, SC). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

Radial chromatography was carried out with a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60  $\text{PF}_{254}$  containing gypsum (E. Merck). Silica gel 60 (40 - 63  $\mu\text{m}$  E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, E. Merck or Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) followed by heating. Dimethylformamide, N-methylformanilide and acetone were dried and stored over activated Linde 4Å molecular sieves under Ar. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. *n*-Hexanes and TMEDA were distilled from sodium under Ar and stored over activated Linde 4Å molecular sieves under Ar. Organic solutions of products were dried over  $\text{MgSO}_4$ .

**Hydrogenation of 2,3-dibenzyloxy-1,4-benzoquinone 9.** A solution of **9** (0.18 g, 0.56 mmol) in EtOH (7.0 mL) was stirred with 10 % Pd on C (0.050 g) at room temperature under  $\text{H}_2$  (1.0 atm) for 3 h. The solution was filtered through Celite® and concentrated to afford a tan solid (0.079 g, 99%) which was identical

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by  $^1\text{H}$  and  $^{13}\text{C}$  NMR to **1** obtained from *myo*-2-inosose. **Hydrogenation of 2,3,4-tribenzyloxyphenol 11**. A solution of **11** (5.8 g, 14.1 mmol) in EtOH (100 mL) was stirred with 10% Pd on C (1.0 g) at room temperature under  $\text{H}_2$  (1.0 atm) for 2 h. The solution was filtered through Celite<sup>®</sup> and concentrated. The residue was  
5 purified by flash chromatography (MeOH/ $\text{CH}_2\text{Cl}_2$ , 1:9, v/v) affording a product which was identical by  $^1\text{H}$  and  $^{13}\text{C}$  NMR to **1** obtained from *myo*-2-inosose.

**1,2,3-Tribenzyloxybenzene 8**. Lowe, W. et al., *Arch. Pharm. (Weinheim)* 327:255 (1994). Benzyl bromide (57 mL, 0.481 mol) and then  $\text{K}_2\text{CO}_3$  (100 g, 0.725 mol) were added to a solution of pyrogallol (20 g, 0.159 mol) in 200 mL dry,  
10 degassed acetone under Ar. This reaction mixture was subsequently stirred for 30 min at room temperature and then for 24 h at reflux under Ar. A solution of NaOH (1.6 g) in MeOH (32 mL) was then added and the reaction refluxed for an additional 30 min. After cooling to room temperature, solids were filtered and washed with acetone. The filtrate was concentrated and the residue recrystallized from MeOH  
15 to afford **8** (52 g, 83%) as an off-white solid. mp 67 - 68 °C.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.44-7.19 (m, 15 H), 6.85 (dd,  $J = 9, 9$  Hz, 1 H), 6.57 (d,  $J = 8.2$  Hz, 2 H), 5.05 (s, 2 H), 5.02 (s, 4 H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  152.9, 138.4, 137.8, 137.0, 128.4, 128.3, 128.0, 127.8, 127.6 (2), 127.3, 127.2, 123.5, 107.7, 75.0, 70.9.

**2,3-Dibenzyloxy-1,4-benzoquinone 9**. To a solution of 1,2,3-  
20 tribenzyloxybenzene **8** (2.0 g, 5.0 mmol) in HOAc (30 mL),  $\text{K}_3\text{Fe}(\text{CN})_6$  (0.82 g, 2.5 mmol) and 30%  $\text{H}_2\text{O}_2$  (1.3 g, 11.5 mmol) were added and the resulting solution stirred at room temperature for 18 h. The solution was diluted with 50 mL  $\text{CH}_2\text{Cl}_2$  and the organic layer subsequently washed with  $\text{H}_2\text{O}$ , saturated aqueous  $\text{NaHCO}_3$  and brine. Drying and concentration resulted in a red oil. Purification by radial  
25 chromatography (2 mm thickness, EtOAc/hexane, 1:19, v/v) afforded **9** as a red oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.36-7.32 (m, 10 H), 6.58 (s, 2 H), 5.20 (s, 4 H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  184.1, 145.2, 136.1, 134.6, 128.5, 128.4, 128.1, 75.1. Anal. Calcd for  $\text{C}_{20}\text{H}_{16}\text{O}_4$ : C, 74.99; H, 5.03. Found: C, 75.04; H, 5.06. HRMS (FAB) calcd for  $\text{C}_{20}\text{H}_{16}\text{O}_4$  ( $\text{M} + \text{H}^+$ ): 320.1049. Found: 320.1059.

**2,3,4-Tribenzyloxybenzaldehyde 10**. Kolonits, P. et al., *Acta Chim. Hung.* 113:367 (1983).  $\text{POCl}_3$  (155 mL, 1.66 mol) was slowly added to *N*-methylformanilide (175 mL, 1.4 mol) at room temperature under Ar which resulted in formation of a yellow solid. After 2 h, the solid was treated with a solution of  
30 1,2,3-tribenzyloxybenzene **8** (20 g, 51 mmol) in anhydrous DMF (40 mL) and  
35 heated to 60 °C. After 3 h, the resulting crimson solution was cooled to room

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temperature and then poured into ice water (3 L) with vigorous stirring for 12 h. The resulting brown precipitate was filtered, washed with hexanes (3 x 100 mL) and finally recrystallized from MeOH to afford **10** (19.8 g, 93%) as a white powder. mp 73 - 74 °C. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 10.11 (s, 1 H), 7.57 (d, *J* = 9 Hz, 1 H), 7.44-7.28 (m, 15 H), 6.83 (d, *J* = 9 Hz, 1 H), 5.21 (s, 2 H), 5.16 (s, 2 H), 5.08 (s, 2 H); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 188.8, 158.5, 155.9, 141.1, 136.9, 136.2, 135.8, 128.6, 128.5 (2), 128.3 (2), 128.2, 127.5, 124.0, 109.1, 76.8, 75.5, 70.9. Anal. Calcd for C<sub>28</sub>H<sub>24</sub>O<sub>4</sub>: C, 79.22; H, 5.70. Found: C, 79.17; H, 5.80. HRMS (FAB) calcd for C<sub>28</sub>H<sub>24</sub>O<sub>4</sub> (M + H<sup>+</sup>): 424.1675. Found: 424.1669.

- 10 **2,3,4-Tribenzyloxyphenol 11.** Kolonits, P. et al., *Acta Chim Hung.* 113:367 (1983). A solution of 30% H<sub>2</sub>O<sub>2</sub> (6 mL, 57.8 mmol) and 85% formic acid (32 mL, 600 mmol) was added dropwise to a solution of 2,3,4-tribenzyloxybenzaldehyde **10** (9.8 g, 23.1 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (50 mL) over 30 min at 0 °C. After 1 h of stirring at 0 °C, the reaction was stirred at room temperature for 24 h. The reaction was  
15 subsequently cooled to 4 °C and diluted with 10% (w/v) aqueous Na<sub>2</sub>SO<sub>3</sub> (50 mL). The aqueous phase was washed with CH<sub>2</sub>Cl<sub>2</sub> (3 x 40 mL). Drying and concentration afforded a brown oil which was dissolved in a methanolic solution of NaOMe (30 mL, 0.1 N) and refluxed. After 10 min, the solution was cooled to 4 °C and acidified with 6 N HCl. MeOH was removed *in vacuo*. The mixture was  
20 diluted with H<sub>2</sub>O (15 mL) followed by extraction of the aqueous phase with benzene (3 x 40 mL). Drying and concentration afforded **11** (9.0 g, 95%) as a brown oil. <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.45 - 7.31 (m, 15 H), 6.65 (d, *J* = 9 Hz, 1 H), 6.58 (d, *J* = 9 Hz, 1 H), 5.28 (s, 1 H), 5.12 (s, 2 H), 5.11 (s, 2 H), 5.04 (s, 2 H). <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 146.0, 144.0, 142.0, 139.6, 137.3, 137.1, 136.8, 128.4, 128.3 (2),  
25 128.2, 127.9, 127.7, 127.4, 110.4, 109.0, 75.6, 75.3, 71.7. Anal. Calcd for C<sub>27</sub>H<sub>24</sub>O<sub>4</sub>: C, 78.62; H, 5.87. Found: C, 78.71; H, 5.86. HRMS (FAB) calcd for C<sub>27</sub>H<sub>24</sub>O<sub>4</sub> (M + H<sup>+</sup>): 412.1675. Found: 412.1673.

### SPECIFIC EXAMPLE 3

#### Synthesis Of Coenzyme Q From 1,2,3,4-Tetrahydroxybenzene

##### 30 I. Results

Variations in strategies employed for hydroxyl protection combined with the ease of metallation and alkylation of the aromatic nucleus makes **1** a versatile intermediate for the synthesis of a wide spectrum of naturally-occurring 1,2,3,4-tetrahydroxybenzene derivatives. For example, permethylation (Figure 5) of **1** leads  
35 to tetramethyl **12** which undergoes facile lithiation and methylation affording **13** in

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high yield. Formation of an organocuprate from **13**, farnesylation, and subsequent reaction with  $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$  affords coenzyme  $\text{Q}_{n=3}$  **4**. This four-step synthesis of coenzyme  $\text{Q}_n$  from tetrahydroxybenzene **1** is equal in length to the shortest reported (Keinan, E. et al., *J. Org. Chem.* 52:3872 (1987)) synthesis of coenzyme  $\text{Q}_n$  which  
5 uses *p*-cresol as a starting material and substantially shorter than syntheses of coenzyme  $\text{Q}_n$  from pyrogallol, gallic acid, or vanillin. Syper, L. et al., *Tetrahedron* 36:123 (1980); Sugihara, H. et al., *Liebigs Ann. Chem.* 763:109 (1972); Mayer, J. et al., *Meth. Enzymol.* 18:182 (1971).

Only one oxygen atom in coenzyme  $\text{Q}_n$ , a shikimate pathway product, is  
10 directly derived from D-glucose. The remaining oxygen atoms are derived from  $\text{O}_2$  via enzyme-catalyzed hydroxylations. Trihydroxybenzenes, pyrogallol, and phloroglucinol possess the maximum number of oxygen atoms attached to a benzene nucleus by the shikimate pathway or polyketide biosynthesis in lieu of enzyme-catalyzed hydroxylation. At least a dozen enzymes are required to  
15 disassemble and reassemble the carbon atoms of D-glucose into the benzene nucleus of coenzyme  $\text{Q}_n$ , pyrogallols, and phloroglucinols. By comparison, synthesis of 1,2,3,4-tetrahydroxybenzene **1** via *myo*-inositol intermediacy requires only four enzymes and an acid-catalyzed dehydration for all six carbon and all four oxygen atoms to be directly derived from the carbon and oxygen atoms of D-  
20 glucose. The method of the present invention for synthesis of 1,2,3,4-tetrahydroxybenzene **1** is thus a useful example of enzyme and atom (Trost, B.M., In *Green Chemistry*, Anastas, P.T., Williamson, T.C. Eds., Oxford: New York, Chap. 6 (1998)) economy in organic synthesis in addition to being a significant strategic departure from previous biocatalytic syntheses of aromatic chemicals from D-  
25 glucose.

## II. Materials And Methods

**General.**  $^1\text{H}$  NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts for  $^1\text{H}$  NMR spectra are reported (in parts per million) relative to internal tetramethylsilane ( $\text{Me}_4\text{Si}$ ,  $\delta = 0.0$  ppm) with  $\text{CDCl}_3$  as solvent, to sodium  
30 3-(trimethylsilyl)propionate-2,2,3,3- $\text{d}_4$  (TSP,  $\delta = 0.0$  ppm) when  $\text{D}_2\text{O}$  was the solvent, and to acetone ( $\text{CHD}_2\text{COCD}_3$ ,  $\delta = 2.04$  ppm) with  $d_6$ -acetone.  $^{13}\text{C}$  NMR spectra were recorded at 75 MHz. Chemical shifts for  $^{13}\text{C}$  NMR spectra are reported (in parts per million) relative to  $\text{CDCl}_3$  ( $\delta = 77.0$  ppm), relative to  $\text{CD}_3\text{COCD}_3$  ( $\delta = 29.8$  ppm), and relative to internal  $\text{CH}_3\text{OH}$  ( $\delta = 49.0$  ppm) or  
35 internal  $\text{CH}_3\text{CN}$  ( $\delta = 1.4$  ppm) in  $\text{D}_2\text{O}$ . FAB mass spectra were performed by

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University of South Carolina (Columbia, SC). Elemental analyses were performed by Atlantic Microlab Inc. (Norcross, GA). Melting points were uncorrected and were determined using a Mel-Temp II melting point apparatus.

Radial chromatography was carried out with a Harrison Associates Chromatotron using 1, 2 or 4 mm layers of silica gel 60 PF<sub>254</sub> containing gypsum (E. Merck). Silica gel 60 (40 - 63  $\mu$ m E. Merck) was used for flash chromatography. Analytical thin-layer chromatography (TLC) utilized precoated plates of silica gel 60 F-254 (0.25 mm, E. Merck or Whatman). TLC plates were visualized by immersion in anisaldehyde stain (by volume: 93% ethanol, 3.5% sulfuric acid, 1% acetic acid and 2.5% anisaldehyde) followed by heating. Dimethylformamide, N-methylformanilide and acetone were dried and stored over activated Linde 4Å molecular sieves under Ar. Tetrahydrofuran and diethyl ether were distilled under nitrogen from sodium benzophenone ketyl. *n*-Hexanes and TMEDA were distilled from sodium under Ar and stored over activated Linde 4Å molecular sieves under Ar. Organic solutions of products were dried over MgSO<sub>4</sub>.

**1,2,3,4-Tetramethoxybenzene 12.** Syper, L. et al., *Tetrahedron* 36:123 (1980). A solution of 1,2,3,4-tetrahydroxybenzene **1** (8.4 g, 59 mmol) and dimethyl sulfate (37.5 mL, 0.396 mol) in EtOH (21 mL) was added dropwise to an 8.5 M aqueous solution of NaOH (42 mL) over 20 min at room temperature. After 2 h, the reaction was diluted with H<sub>2</sub>O (300) mL and cooled to -20 °C for 12 h. The resulting precipitate was filtered, washed with H<sub>2</sub>O, and then recrystallized from hexanes to afford **12** (8.12 g, 69%) as colorless needles. mp 84 - 85 °C. <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  6.58 (s, 2 H), 3.90 (s, 6 H) 3.82 (s, 6 H). <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$  147.7, 143.3, 106.3, 61.1, 56.3. Anal. Calcd for C<sub>10</sub>H<sub>14</sub>O<sub>4</sub>: C, 60.59; H, 7.12. Found: C, 60.44; H, 7.07.

**2,3,4,5-Tetramethoxytoluene 13.** Syper, L. et al., *Tetrahedron* 36:123 (1980). To a solution of 1,2,3,4-tetramethoxybenzene **12** (4.0 g, 20.2 mmol) and TMEDA (6 mL, 38.0 mmol) in hexanes (44 mL) and THF (80 mL) at 0 °C under Ar, *n*-BuLi in hexane (1.6 M, 25.6 mmol) was added dropwise over a 10 min period and the reaction stirred for 30 min at 0 °C under Ar. Subsequent to dropwise addition of CH<sub>3</sub>I (20 mL, 160 mmol) over an 8 min period, the reaction was stirred for 3 h at 0 °C under Ar and then quenched by addition of aqueous NH<sub>4</sub>Cl and ether (20 mL). The organic layer was sequentially washed with concentrated NH<sub>4</sub>OH, water, and brine. Drying and concentration of the organic layer was followed by purification of the residue by flash chromatography (hexanes, hexanes/EtOAc, 19:1,

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v/v) to afford **13** as a clear oil (3.6 g, 83%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  6.45 (s, 1 H), 3.93 (s, 3 H), 3.87 (s, 3 H), 3.82 (s, 3 H), 3.79 (s, 3 H), 2.23 (s, 3 H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  149.0, 146.9, 145.3, 140.7, 125.7, 108.2, 61.0, 60.9, 60.5, 55.9, 15.7.

**Protected coenzyme  $Q_3$  14.** Keinan, E. et al., *J. Org. Chem.* 52:3872 (1987).  $n\text{-BuLi}$  (1.6 M, 0.9 mL) was added dropwise over a 15 min period to a solution of 1,2,3,4-tetramethoxytoluene **13** (0.212 g, 1 mmol) and TMEDA (0.3 mL, 1.9 mmol) in hexane (2.2 mL) at 0 °C under Ar. This yellow precipitate-containing reaction mixture was then stirred at 0 °C under Ar for 30 min, diluted with THF (4 mL) and ether (11 mL), followed by addition of CuCN (0.125 g, 1.4 mmol). After stirring for 30 min at 0 °C under Ar, the temperature was reduced to -78 °C, and a solution of farnesyl bromide (0.285 g, 1 mmol) in hexane (2 mL) was dropwise added over a 30 min period. Further reaction for 3 h at -78 °C and subsequent slow warming to room temperature was followed by addition of saturated aqueous  $\text{NH}_4\text{Cl}$  (10 mL) and ether (20 mL). Washing the organic phase with concentrated  $\text{NH}_4\text{OH}$ , water, and brine was followed by drying and concentration. Purification of the residue by radial chromatography (2 mm thickness, hexane/EtOAc, 9:1, v/v) afforded **14** as a clear oil (0.236 g, 57%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.12-5.01 (m, 3 H), 3.90 (s, 6 H), 3.78 (s, 6 H), 3.32 (d,  $J = 7$  Hz, 2 H), 2.14 (s, 3 H), 2.08-1.91 (m, 8 H), 1.77 (s, 3 H), 1.66 (s, 3 H), 1.58 (s, 6 H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  147.8, 147.6, 144.9, 144.6, 135.0, 134.9, 131.2, 129.2, 125.4, 124.3, 124.1, 122.8, 61.1, 60.6, 39.7, 26.7, 26.5, 25.7, 25.6, 17.6, 16.2, 15.9, 11.7.

**Coenzyme  $Q_3$  4.** Keinan, E. et al., *J. Org. Chem.* 52:3872 (1987). A suspension maintained at 0 °C resulting from addition of pyridine-2,6-dicarboxylate (0.125 g, 0.75 mmol) to a solution of protected coenzyme  $Q_3$  **14** in  $\text{CH}_3\text{CN}$  (1.4 mL) and water (0.6 mL) at 0 °C was reacted with a 0 °C solution of  $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$  (0.411 g, 0.75 mmol) in  $\text{CH}_3\text{CN}$  (0.4 mL) and water (0.4 mL) added dropwise over a 10 min period. After 40 min at 0 °C, the reaction was warmed to room temperature and stirred for 20 min. Water (10 mL) was added to the reaction mixture and the resulting solution extracted with  $\text{CH}_2\text{Cl}_2$  (3x 100 mL). The combined organic phases were dried, concentrated, and purified by radial chromatography (1 mm thickness, hexane/EtOAc, 19:1, v/v) to afford **4** (0.053 g, 46%) as an orange oil.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.07 (dd,  $J = 7, 7$  Hz, 1 H), 5.05 (dd,  $J = 7, 7$  Hz, 1 H), 4.94 (dd,  $J = 7, 7$  Hz, 1 H), 3.99 (s, 3 H), 3.98 (s, 3 H), 3.18 (d,  $J = 6.8$  Hz, 2 H), 2.08-1.91 (m, 8 H), 2.01 (s, 3 H), 1.74 (s, 3 H), 1.67 (s, 3

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H), 1.59 (s, 3 H), 1.58 (s, 3 H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  184.7, 183.9, 144.3, 144.2, 141.6, 138.8, 137.6, 135.2, 131.3, 124.3, 123.8, 118.8, 61.1, 39.7, 26.7, 26.4, 25.7, 25.3, 17.6, 16.3, 16.0, 11.9; HRMS (FAB) calcd for  $\text{C}_{24}\text{H}_{34}\text{O}_4$  ( $\text{M} + \text{H}^+$ ): 386.2457. Found: 386.2461

5

#### SPECIFIC EXAMPLE 4

##### Synthesis Of *Myo*-2-Inosose By A Single Microbe

*Myo*-2-inosose (1 g/L), *myo*-inositol (18 g/L), and *myo*-inositol-1-phosphate (3.1 g/L) were synthesized by *E. coli* JWF1/pAD2.28A in 9.6% (mol/mol) yield from glucose under fed-batch fermentation conditions as described in Specific Example

10 1. The fermentation ran for 54 h with incremental addition of IPTG (0.0048 g added each time) at 7 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, and 54 h. The *myo*-2-inosose can then be converted to 1,2,3,4-tetrahydroxybenzene by acid-catalyzed dehydration as described in Specific Example 1.

Plasmid pAD2.28A carries *INO1* under  $P_{tac}$  promoter control, *iolG* under  $P_{lac}$  promoter control, *lacI<sup>q</sup>*, and *serA*. *INO1* encodes *myo*-inositol-1-phosphate synthase and was cloned out of *Saccharomyces cerevisiae*. Inositol dehydrogenase is encoded by the *iolG* locus, which was cloned out of *Bacillus subtilis*.

15

#### SPECIFIC EXAMPLE 5

##### Conversion Of 1,2,3,4-Tetrahydroxybenzene To Pyrogallol

20 A solution consisting of 0.4 g of NaOH (10 mmol) dissolved in 10 mL  $\text{H}_2\text{O}$  was freeze-thaw degassed three times under Ar. This solution was then added via cannula under Ar to a 250 mL Parr bottle containing 1,2,3,4-tetrahydroxybenzene (1.42 g, 10 mmol) and 5% Rh/ $\text{Al}_2\text{O}_3$  (0.25 g), which had been flushed with Ar and then sealed with a septum. The resulting red/brown solution was hydrogenated

25 under 50 psi.  $\text{H}_2$  using a Parr Hydrogenator. After 12 h, the solution was filtered through Celite® and the catalyst rinsed with 10 mL  $\text{H}_2\text{O}$ . The resulting dark brown solution was adjusted to pH = 6.0 with 10% HCl and then concentrated to a brown oil. The oil was dissolved in 50 mL 0.5 M  $\text{H}_2\text{SO}_4$  that had been degassed by aeration with Ar for 20 minutes. The solution was heated to reflux under Ar for

30 12 h, cooled to room temperature, and then extracted with  $\text{Et}_2\text{O}$  (4 x 50 mL). The combined organic layers were dried over  $\text{MgSO}_4$ , filtered and concentrated to a brown oil. Kugel-Rohr distillation of the oil under vacuum (1 mm Hg) at 90 °C afforded 0.56 g pyrogallol (4.44 mmol, 44% yield) as a white, crystalline solid.

The foregoing discussion discloses and describes merely exemplary  
35 embodiments of the present invention. One skilled in the art will readily recognize

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from such discussion, and from the accompanying drawings and claims, that various changes, modifications and variations can be made therein without departing from the spirit and scope of the invention as defined in the following claims.

- 5 All references cited herein are incorporated by reference as if fully set forth.



**WE CLAIM:**

1. A method for the production of 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source, comprising the steps of:
  - a) converting the carbon source to *myo*-inositol with a first recombinant  
5 microbe;
  - b) converting the *myo*-inositol to *myo*-2-inosose with a second microbe; and
  - c) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.
- 10 2. The method of Claim 1, wherein the first recombinant microbe comprises the gene encoding *myo*-inositol-1-phosphate synthase.
3. The method of Claim 2, wherein the gene encoding *myo*-inositol-1-phosphate synthase is a *S. cerevisiae INO1* gene.
4. The method of Claim 3, wherein the *S. cerevisiae INO1* gene is  
15 located on a plasmid in the recombinant microbe.
5. The method of Claim 4, wherein the plasmid is pAD1.88A.
6. The method of Claim 1, wherein the first recombinant microbe is *E. coli* JWF1/pAD1.88A.
7. The method of Claim 1, wherein the second microbe expresses  
20 inositol dehydrogenase activity.
8. The method of Claim 1, wherein the second microbe is *Gluconobacter oxydans*.
9. The method of Claim 8, wherein the *Gluconobacter oxydans* is *Gluconobacter oxydans* ATCC 621.
- 25 10. The method of Claim 1, wherein the second microbe is a recombinant microbe comprising the gene for inositol dehydrogenase.

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11. The method of Claim 10, wherein the gene for inositol dehydrogenase is a *Bacillus subtilis iolG* gene.

12. The method of Claim 1, wherein the carbon source is glucose.

13. The method of Claim 1, further comprising the step of reducing the  
5 1,2,3,-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

14. A method of producing 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source comprising the steps of:

- a) converting the carbon source to *myo*-inositol with a recombinant *E. coli* comprising the gene encoding *myo*-inositol-1-phosphate synthase;
- 10 b) converting the *myo*-inositol to *myo*-2-inosose with a microbe that expresses inositol dehydrogenase activity; and
- c) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

15. The method of Claim 14, wherein the gene encoding *myo*-inositol-1-phosphate synthase is the *S. cerevisiae INO1* gene.

16. The method of Claim 15, wherein the *INO1* gene is located on a plasmid in the recombinant *E. coli*.

17. The method of Claim 16, wherein the plasmid is pAD1.88A.

18. The method of Claim 14, wherein the recombinant *E. coli* is *E. coli*  
20 JWF1/pAD1.88A.

19. The method of Claim 14, wherein the carbon source is glucose.

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20. A method for the production of 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source, comprising the steps of:

a) converting the carbon source to *myo*-2-inosose with a recombinant microbe; and

5 b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

21. The method of Claim 20, wherein the recombinant microbe comprises the genes encoding for *myo*-inositol-1-phosphate synthase and inositol dehydrogenase.

10 22. The method of Claim 21, wherein the gene encoding for *myo*-inositol-1-phosphate synthase is a *Saccharomyces cerevisiae* INO1 gene.

23. The method of Claim 22, wherein the INO1 gene is located on a plasmid in the recombinant microbe.

24. The method of Claim 23, wherein the plasmid is pAD2.28A.

15 25. The method of Claim 21, wherein the gene encoding for inositol dehydrogenase is an *iolG* gene.

26. The method of Claim 25, wherein the *iolG* gene is a *Bacillus subtilis* *iolG* gene.

20 27. The method of Claim 25, wherein the *iolG* gene is located on a plasmid in the recombinant microbe.

28. The method of Claim 27, wherein the plasmid is pAD2.28A.

29. The method of Claim 20, wherein the recombinant microbe is JWF1/pAD2.28A.

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30. A method of producing 1,2,3,4-tetrahydroxybenzene and derivatives thereof from a carbon source comprising the steps of:

- a) converting the carbon source to *myo*-2-inosose with a recombinant *E. coli* comprising the genes encoding for *myo*-1-phosphate synthase and inositol  
5 dehydrogenase; and
- b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid catalyzed dehydration.

31. The method of Claim 30, wherein the gene encoding for *myo*-inositol-1-phosphate synthase is the *S. cerevisiae INO1* gene.

10 32. The method of Claim 30, wherein the gene encoding for inositol dehydrogenase is the *Bacillus subtilis iolG* gene.

33. The method of Claim 30, wherein the recombinant *E. coli* is JWF1/pAD2.28A.

15 34. A method for the production of 1,2,3-trihydroxybenzene and derivatives thereof from a carbon source, comprising the steps of:

- a) converting the carbon source to *myo*-inositol with a first recombinant microbe;
- b) converting the *myo*-inositol to *myo*-2-inosose with a second microbe;
- c) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid  
20 catalyzed dehydration; and
- d) reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene.

35. The method of Claim 34, wherein the first recombinant microbe comprises the gene encoding *myo*-inositol-1-phosphate synthase.

25 36. The method of Claim 35, wherein the gene encoding *myo*-inositol-1-phosphate synthase is the *S. cerevisiae INO1* gene.

37. The method of Claim 36, wherein the *S. cerevisiae INO1* gene is located on a plasmid in the recombinant microbe.

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38. The method of Claim 37, wherein the plasmid is pAD1.88A.

39. The method of Claim 34, wherein the first recombinant microbe is *E. coli* JWF1/pAD1.88A.

40. The method of Claim 34, wherein the second microbe expresses  
5 inositol dehydrogenase activity.

41. The method of Claim 34, wherein the second microbe is  
*Gluconobacter oxydans*.

42. The method of Claim 41, wherein the *Gluconobacter oxydans* is  
*Gluconobacter oxydans* ATCC 621.

10 43. The method of Claim 34, wherein the carbon source is glucose.

44. The method of Claim 34, wherein the 1,2,3,4-tetrahydroxybenzene  
is reduced to 1,2,3-trihydroxybenzene by catalytic hydrogenation and acid catalyzed  
hydrolysis.

15 45. The method of Claim 44, wherein the catalytic hydrogenation uses  
Rh/Al<sub>2</sub>O<sub>3</sub> as the catalyst.

46. A method for the production of 1,2,3-trihydroxybenzene and  
derivatives thereof from a carbon source, comprising the steps of:

- a) converting the carbon source to *myo*-2-inosose with a recombinant  
microbe;
- 20 b) converting the *myo*-2-inosose to 1,2,3,4-tetrahydroxybenzene by acid  
catalyzed dehydration; and
- c) reducing the 1,2,3,4-tetrahydroxybenzene to 1,2,3-  
trihydroxybenzene.

25 47. The method of Claim 46, wherein the recombinant microbe comprises  
the genes encoding for *myo*-inositol-1-phosphate synthase and inositol  
dehydrogenase.

48. The method of Claim 47, wherein the gene encoding for *myo*-inositol-1-phosphate synthase is a *Saccharomyces cerevisiae* INO1 gene.

49. The method of Claim 48, wherein the INO1 gene is located on a plasmid in the recombinant microbe.

5 50. The method of Claim 49, wherein the plasmid is pAD2.28A.

51. The method of Claim 47, wherein the gene encoding for inositol dehydrogenase is an *iolG* gene.

52. The method of Claim 51, wherein the *iolG* gene is a *Bacillus subtilis* *iolG* gene.

10 53. The method of Claim 51, wherein the *iolG* gene is located on a plasmid in the recombinant microbe.

54. The method of Claim 53, wherein the plasmid is pAD2.28A.

55. The method of Claim 46, wherein the recombinant microbe is JWF1/pAD2.28A.

15 56. The method of Claim 34, wherein the 1,2,3,4-tetrahydroxybenzene is converted to 1,2,3-trihydroxybenzene by catalytic hydrogenation and acid catalyzed hydrolysis.

57. The method of Claim 56, wherein the catalytic hydrogenation uses Rh/Al<sub>2</sub>O<sub>3</sub> as the catalyst.

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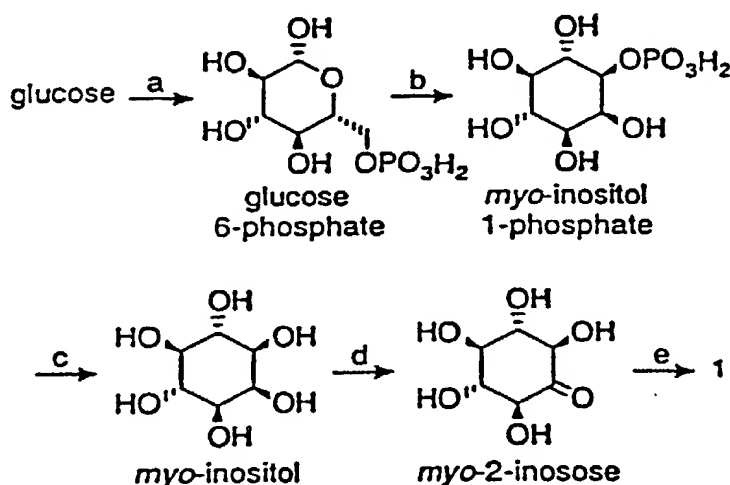
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant (for all designated States except US): BOARD OF TRUSTEES OPERATING MICHIGAN STATE UNIVERSITY [US/US]; East Lansing, MI 48824 (US).  (72) Inventors; and (75) Inventors/Applicants (for US only): FROST, John, W. [US/US]; 1621 Dobie Circle, Okemos, MI 48864 (US). HANSEN, Chad, A. [US/US]; Apt. 2B, 605 Glenmoor Road, East Lansing, MI 48823 (US).  (74) Agents: GIBBS, Barbara, S. et al.; Harness, Dickey & Pierce, P.L.C., P.O. Box 828, Bloomfield Hills, MI 48303 (US).			

(54) Title: SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1,2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1-PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DEHYDROGENASE



<sup>a</sup>Key: (a) phosphoenolpyruvate:carbohydrate phosphotransferase; (b) myo-inositol 1-phosphate synthase; (c) phosphatase activity; (d) dehydrogenase activity; (e) 0.5 M H<sub>2</sub>SO<sub>4</sub>, H<sub>2</sub>O, reflux.

(57) Abstract

A bioengineered synthesis scheme for the production of 1,2,3,4-tetrahydroxybenzene from a carbon source is provided. Methods of producing 1,2,3,4-tetrahydroxybenzene acid from a carbon source based on the synthesis scheme are also provided. Methods are also provided for converting 1,2,3,4-tetrahydroxybenzene to 1,2,3-trihydroxybenzene by catalytic hydrogenation.

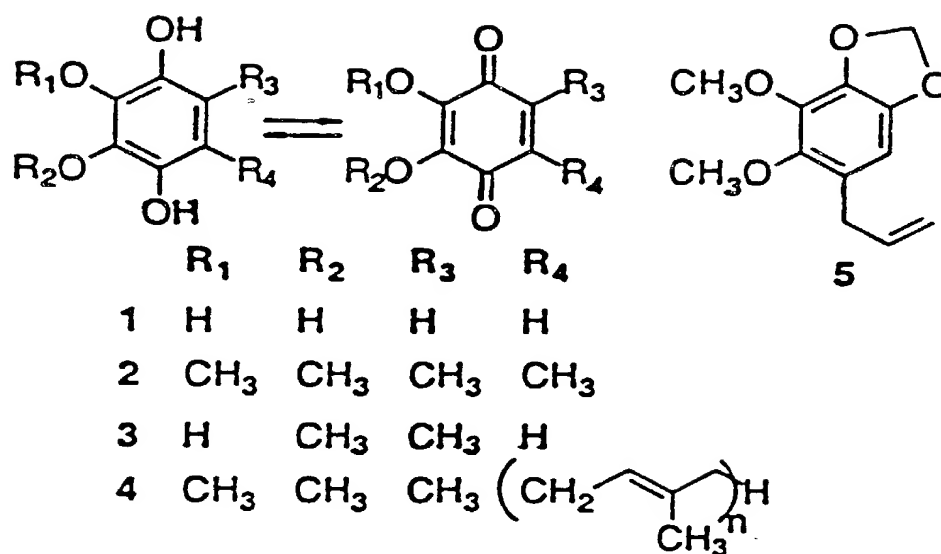
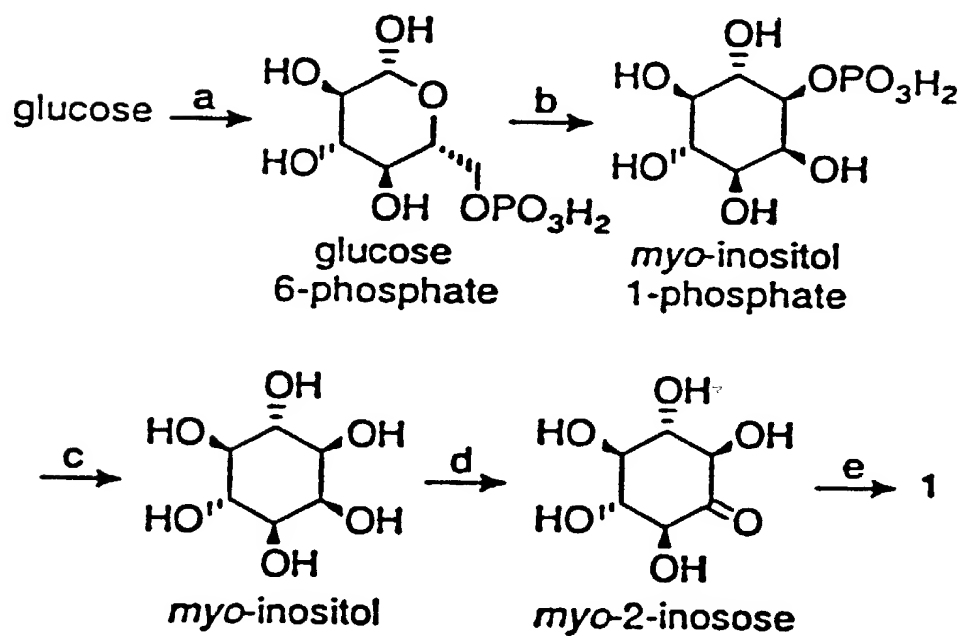


FIGURE 1



<sup>a</sup>Key: (a) phosphoenolpyruvate:carbohydrate phosphotransferase; (b) *myo*-inositol 1-phosphate synthase; (c) phosphatase activity; (d) dehydrogenase activity; (e) 0.5 M H<sub>2</sub>SO<sub>4</sub>, H<sub>2</sub>O, reflux.

FIGURE 2



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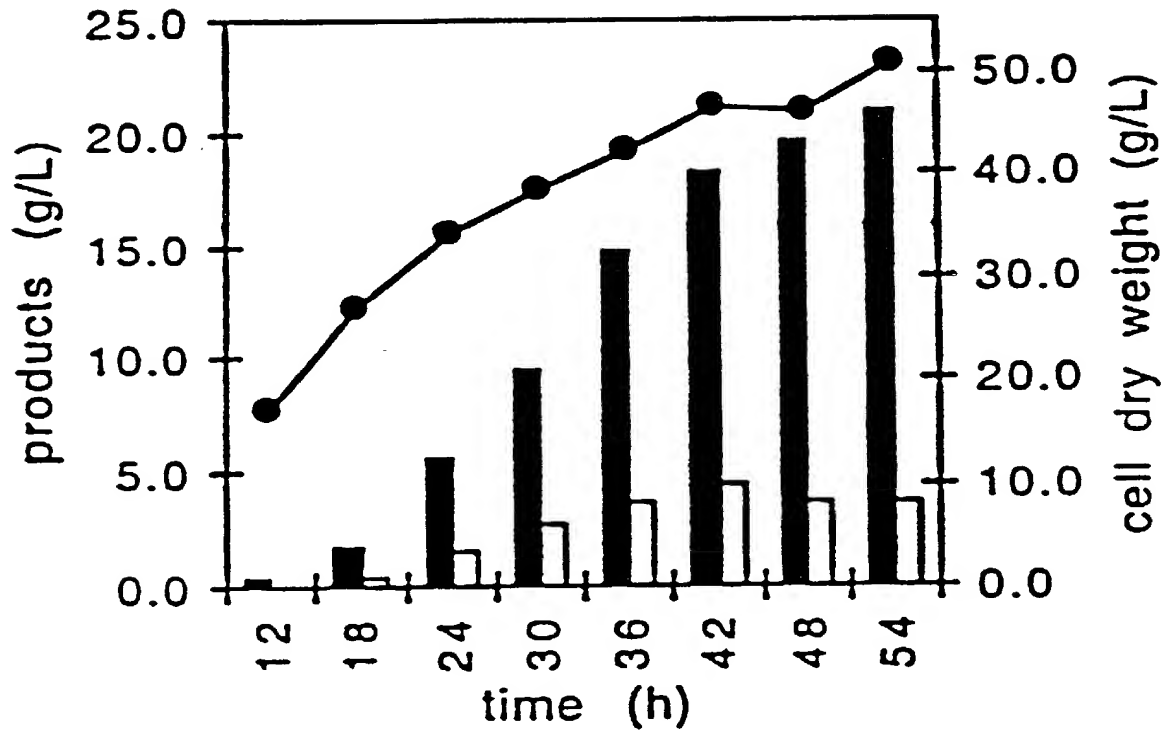
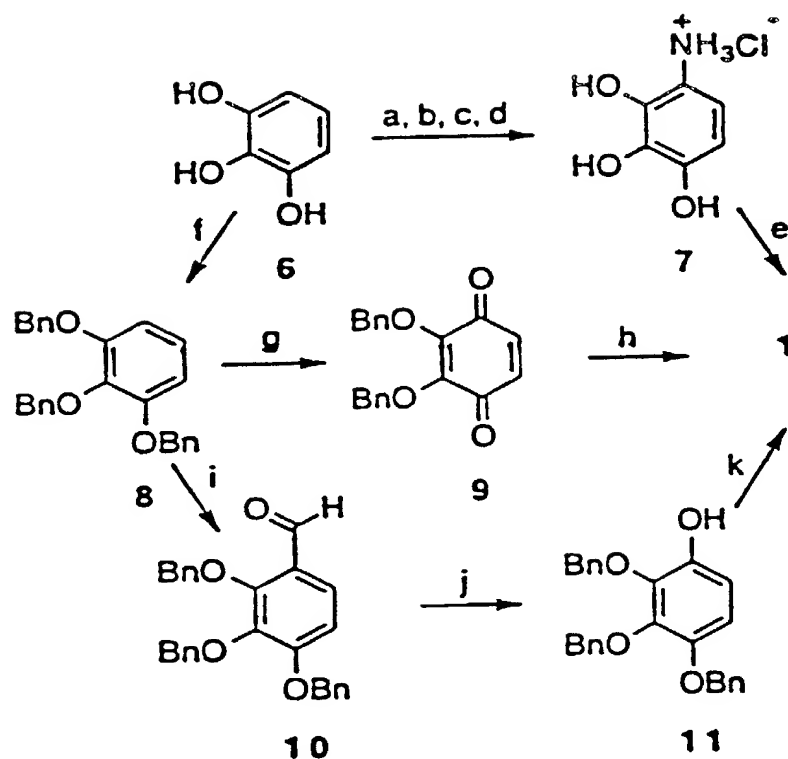


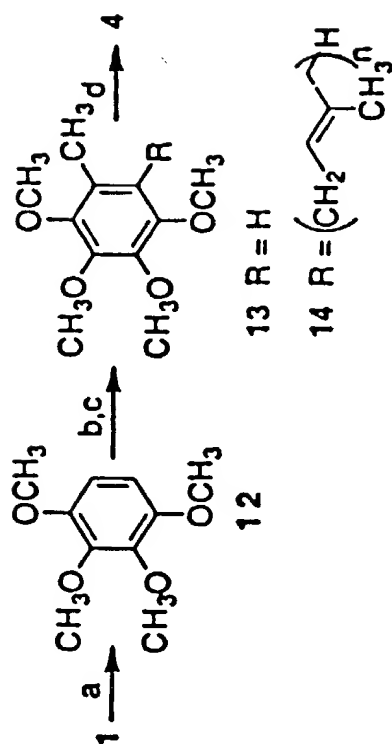
FIGURE 3



<sup>a</sup>Key: (a)  $\text{Cl}_2\text{C}(\text{O})$ , pyridine, xylene, reflux; (b)  $\text{H}_2\text{SO}_4$ ,  $\text{HNO}_3$ ; (c)  $\text{KOH}$  (aq.); (d)  $\text{Zn}$ ,  $\text{HCl}$ ; (e)  $\text{H}_2\text{O}$ , reflux; (f)  $\text{BnBr}$ ,  $\text{K}_2\text{CO}_3$ , acetone, reflux, 83 %; (g)  $\text{K}_3\text{Fe}(\text{CN})_6$ ,  $\text{H}_2\text{O}_2$ ,  $\text{AcOH}$ , 11 %; (h)  $\text{H}_2$ , 10 %  $\text{Pd/C}$ ,  $\text{EtOH}$ , 100 %; (i) *N*-methylformanilide,  $\text{POCl}_3$ , 60 °C, 93 %; (j)  $\text{HCO}_2\text{H}$ ,  $\text{H}_2\text{O}_2$ ,  $\text{CH}_2\text{Cl}_2$ , 0 °C to rt, 95 %; (k)  $\text{H}_2$ , 10 %  $\text{Pd/C}$ ,  $\text{EtOH}$ , 80%.

FIGURE 4

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<sup>a</sup>Key: (a)  $(\text{CH}_3)_2\text{SO}_4$ , NaOH, 69 %; (b) (i) *n*-BuLi, TMEDA, hexanes, THF, 0 °C; (ii)  $\text{CH}_3\text{I}$ , 0 °C, 83%; (c) (i) *n*-BuLi, TMEDA, hexanes, 0 °C; (ii) CuCN, THF,  $\text{Et}_2\text{O}$ , 0 °C; (iii) famesyl bromide, -78 °C, 57 %; (d) CAN, pyridine-2,6-dicarboxylate,  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ , 0 °C, 46%.

FIGURE 5

# DECLARATION AND POWER OF ATTORNEY

Atty. Dkt. No.: 6550-000038/USB

## DECLARATION

As a below named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name,

I believe that I am the original and first inventor or inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

### SYNTHESIS OF 1,2,3,4-TETRAHYDROXYBENZENES AND 1,2,3-TRIHYDROXYBENZENES USING MYO-INOSITOL-1-PHOSPHATE SYNTHASE AND MYO-INOSITOL 2-DEHYDROGENASE

the specification of which (check one)

- ☐ is attached hereto.  
or  
☒ was filed on September 21, 2001 as Application Serial No. or PCT International Application No. 09/937,243 and was amended on September 21, 2001 and October 31, 2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.


I hereby claim foreign priority benefits under 35 U.S.C. §§ 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)				
APPN. SERIAL NO.	COUNTRY	DATE FILED (MM/DD/YYYY)	PRIORITY CLAIM	
			Yes	No
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>
			<input type="checkbox"/>	<input type="checkbox"/>


## DECLARATION AND POWER OF ATTORNEY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

### POWER OF ATTORNEY

I hereby appoint each practitioner at Customer No. 27572 (  ) of Harness, Dickey & Pierce, P.L.C., my attorney with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

### CORRESPONDENCE ADDRESS

I request the Patent and Trademark Office to direct all correspondence and telephone calls relative to this application to Customer No. 27572 (  ), Harness, Dickey & Pierce, P.L.C., P. O. Box 828, Bloomfield Hills, Michigan 48303 (248) 641-1600.

DECLARATION AND POWER OF ATTORNEY

Docket No. 6550-000038/USB

Serial No. 09/937,243

✓ **Full name of sole or first inventor:** John W. Frost

Inventor's signature: John W. Frost

Date: 3/04/02

Residence: 1621 Dobie Circle, Okemos, Michigan MI 48864

Citizenship: United States of America

Mailing Address: same as above

2/ **Full name of second joint inventor, if any:** Chad A. Hansen

Inventor's signature: Chad A. Hansen

Date: 3/4/02 MI

Residence: 605 Glenmoor Road, Apartment 2B, East Lansing, Michigan 48823

Citizenship: United States of America

Mailing Address: same as above